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(57) Abstract

The invention relates to peptides comprising an amino acid sequence substantially homologous to the amino sequence of a fragment of a pyrogenic exotoxin, and functional derivatives of said peptides, capable of eliciting protective immunity against toxic shock induced by a pyrogenic exotoxin or by a mixture of pyrogenic exotoxins. Preferred peptides comprise an amino acid sequence substantially homologous to the amino sequence of a fragment of *Staphylococcal aureus* enterotoxin B (SEB). The invention also relates to broad spectrum pharmaceutical compositions for the treatment or short term prophylaxis of toxin-mediated activation of T cells, comprising as active ingredient at least one peptide according to the invention or derivative thereof, and to broad spectrum vaccines for conferring long term immunity against toxic shock induced by at least one pyrogenic exotoxin are provided. The vaccines comprising as active ingredient at least one peptide according to the invention, or derivative thereof.

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## BROAD SPECTRUM PYROGENIC EXOTOXINS ANTAGONISTS AND VACCINES

### FIELD OF THE INVENTION

5 The invention relates to peptides structurally related to protein domains in pyrogenic exotoxins, capable of antagonizing activation of T cells mediated by said exotoxins and of eliciting protective immunity against toxic shock induced by said exotoxins. The invention further relates to pharmaceutical preparations containing the said peptides, for the treatment or prophylaxis of toxic shock and to vaccines containing  
10 the said peptides, capable of eliciting protective immunity against toxic shock induced by said exotoxins.

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15 Department of the Army.

### BACKGROUND OF THE INVENTION

A family of pyrogenic exotoxins, also known as superantigenic toxins, is produced by *Staphylococcus aureus* and *Streptococcus pyogenes*. The exotoxins comprised of the *S. aureus* enterotoxins (SEs) cause the majority of human food poisoning cases  
20 manifested by vomiting and diarrhea after ingestion [Schlievert, J Infect Dis 167:997 (1993)]. *S. aureus* is found widespread in nature, often in association with humans. Among the 5 major serological types within the family of SEs (labeled SEA to SEE and SEG), SEB is the most prominent [Marrack and Kappler, Science 248:705 (1990)]. SEB has also been recognized as a leading cause of human cases  
25 of non-menstrual toxic shock syndrome that can accompany surgical or injurious wound infections, as well as viral infections of the respiratory tract of influenza patients to which children are especially vulnerable [Schlievert (1993) *ibid.*; Tseng *et al.*, Infect Immun 63:2880 (1995)]. Toxic shock syndrome, in its most severe form, causes shock and death [Murray *et al.*, ASM News 61:229 (1995); Schlievert  
30 (1993) *ibid.*]. More generally, members of the staphylococcal exotoxin family, including SEA to SEE and toxic shock syndrome toxin 1 (TSST-1), have been implicated in toxic shock syndrome, in atopic dermatitis [Schlievert (1993) *ibid.*] and in Kawasaki's syndrome [Bohach *et al.*, Crit Rev Microbiol 17:251 (1990)].

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- Because of the potential for causing lethal shock in humans after aerosol exposure and because of the relative ease with which SEB can be produced in large amounts, there is concern that SEB could be used as a biological weapon [Lowell *et al.*, Infect Immun **64**:1706 (1996)]. SEB is thought to be a biological weapon mainly in view of its lethal potential. However, through its exquisite ability to induce vomiting and diarrhea, SEB is also an incapacitating agent that could severely impair the effectiveness of a fighting force, even temporarily, thereby enhancing vulnerability to conventional military means. Needless to say, the harmful effects of SEB need to be generally attacked, and not only in connection with the military aspect.
- SEB is a toxic mitogen that triggers a paradoxical response in the infected organism: a vast stimulation of the immune system on one hand side and, on the other hand, a profound immunosuppression that may allow the multiplication of the infecting bacteria, unimpeded by an immune response [Hoffman, Science **248**:685 (1990); Smith and Johnson J Immunol **115**:575 (1975); Marrack *et al.*, J Exp Med **171**:455 (1990); Pinto *et al.*, Transplantation **25**:320 (1978)]. During the cellular immune response, a dynamic interplay is induced, by antigens or mitogens, between activation of Th1 type cytokine gene expression on one hand, exemplified by interleukin-2 (IL-2), interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\beta$  (TNF- $\beta$ ), and on the other hand, its cell-mediated suppression by CD8 cells and other cell subsets [Ketzinel *et al.*, Scand J Immunol **33**:593 (1991); Arad *et al.*, Cell Immunol **160**:240 (1995)], and by the inhibitory cytokines from Th2 cells, IL-4 and IL-10 [Mosmann and Coffman, Annu Rev Immunol **7**:145 (1989)].
- SEB is a member of the family of pyrogenic exotoxins [Herman *et al.*, Ann Rev Immunol **9**:745 (1991)] that comprises bacterial exotoxins and MIs proteins. These agents stimulate a 20,000-fold greater proportion of rodent or human T cells than do ordinary antigens. Thus, SEB activates 30-40% of all T cells in some mice to divide and produce cytokines [Marrack and Kappler (1990) *ibid.*]. Indeed, expression of the toxicity of SEB requires T cells; mice that lack T cells or SEB-reactive T cells are not affected by doses of SEB that cause weight loss and death in normal animals [Marrack *et al.* (1990) *ibid.*; Marrack and Kappler (1990) *ibid.*]. Unlike normal antigens, SEB and related toxic mitogens do not require processing and antigen presentation [Janeway *et al.*, Immunol Rev **107**:61 (1989)] but activate the T cell by binding at a specific site in the variable portion of the  $\beta$  chain (V- $\beta$ ) of the T-cell receptor [Choi *et al.*, Nature **346**:471 (1990)]. The crucial region for T-cell receptor interaction with toxin lies on the outer face of the V- $\beta$  domain, a region not involved in conventional antigen recognition [Choi *et al.*, Proc Natl Acad Sci U.S.A. **86**:8941

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(1989)]. Simultaneously, pyrogenic exotoxins bind directly to MHC class II molecules [Scholl *et al.*, Proc Natl Acad Sci U.S.A. **86**:4210 (1989)] and thus affect primarily CD4<sup>+</sup> T cells, although CD8<sup>+</sup> cells are also activated [Fleischer and Schrezenmeier, J Exp Med **167**:1697 (1988); Fraser, Nature **339**:221 (1989); Misfeldt, Infect Immun **58**:2409 (1990)]. The current consensus is that pyrogenic exotoxins activate T cells so effectively because they bypass the ordinary interaction of antigen with class II MHC and T-cell receptor [Janeway, Cell **63**:659 (1990)]. An alternative view is that pyrogenic exotoxins act as coligands that facilitate and thus greatly exaggerate the effect of minute amounts of ordinary antigens [Janeway (1990) *ibid.*].

The toxicity of SEB and related exotoxins is thought to be related to the capacity of these molecules to stimulate the rapid and excessive production of cytokines, especially of IL-2, IFN- $\gamma$  and tumor necrosis factors (TNFs). IL-2, IFN- $\gamma$ , and TNF- $\beta$  are secreted from activated T helper type 1 (Th1) cells while TNF- $\alpha$  is secreted by Th1 cells, monocytes and macrophages. High levels of these cytokines, suddenly produced, have been implicated as a central pathogenic factor in toxin-related toxicity [Schad *et al.*, EMBO J **14**:3292 (1995)] and are thought to cause a rapid drop in blood pressure leading to toxic shock.

While investigation has produced a plausible explanation for the vast stimulation of T cells by SEs, it is not yet clear why these toxins are also strongly immunosuppressive. They induce a decline in both primary T and B cell responses, including the production of antibodies and the generation of plaque-forming cells [Hoffman (1990) *ibid.*; Smith and Johnson (1975) *ibid.*; Marrack *et al.* (1990) *ibid.*; Pinto *et al.* (1978) *ibid.*; Ikejima *et al.*, J Clin Invest **73**:1312 (1984); Poindexter and Schlievert, J Infect Dis **153**:772 (1986)].

The sensitivity of humans to staphylococcal toxins exceeds that of mice by a factor of 100. Thus, the toxic shock syndrome toxin 1, TSST-1, another pyrogenic exotoxin from *Staphylococcus aureus*, stimulates human T cells to express the key cytokines, IL-2, IFN- $\gamma$  and TNF- $\beta$  at <0.1 pg/ml, while murine cells require approximately 10 pg/ml [Uchiyama *et al.*, J Immunol **143**:3173 (1989)]. Mice may have developed relative resistance to toxic mitogens by deleting from their T cell repertoire those cells that display the most highly reactive V- $\beta$  chains or by eliminating these V- $\beta$  genes [Marrack and Kappler (1990) *ibid.*]. Such deletions have not been detected in humans, making them far more vulnerable.

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The incapacitating and potentially lethal effects for humans of SEB (and of exotoxins of the same family of superantigens), whether exerted on civilians or military personnel, create a need for prophylaxis against SEB, for treatment of SEB-exposed individuals and for a safe SEB vaccine.

- 5 Despite the urgency of this need, methods of protection or treatment have been lacking. Thus, in *D*-galactosamine-sensitized murine models of SEB intoxication, one based on intramuscular challenge with SEB toxin and the other on intranasal challenge using mucosal SEB exposure, it was possible to protect mice with proteosome-SEB toxoid vaccines in which the SEB toxoid component was prepared  
10 by a 30-day formalin treatment of the biologically active, intact SEB protein molecule [Lowell *et al.* (1996) *ibid.*]. As detailed below, however, the inventors have now found that antibodies raised against certain peptide domains within the SEB molecule enhance the ability of SEB to stimulate human T cells, rather than protecting them against the toxin. This finding limits the use of SEB toxoids as  
15 vaccine, in view of the danger of eliciting certain SEB-sensitizing antibodies that could not only fail to confer protective immunity but would lead to significant exacerbation of the toxic responses in SEB-exposed persons.

Other investigators sought recourse in the use of fragments rather than the complete SEB protein molecule, through the synthesis of a series of overlapping SEB  
20 peptides, in the order of 30 amino acids each in length [Jett *et al.*, *Infect Immun* 62:3408 (1994)]. These peptides were used to generate antisera in rabbits whose ability to inhibit the SEB-induced proliferation of a mixture of human T cells and macrophages was then tested. That effort failed to yield an effective or specific inhibitory response. Thus, peptide pSEB(113-144), containing amino acids 113 to  
25 144 of the SEB protein molecule, as well as peptides covering amino acids 130-160, 151-180, and 171-200 each elicited antisera that inhibited the SEB-induced lymphocyte proliferation weakly, by up to 2.5-fold [Jett *et al.* (1994) *ibid.*].

A number of investigators attempted the create peptide vaccines. Thus, Mayordomo  
30 *et al.* [*J Exp Med* 183:1357 (1996)] used a mutant peptide derived from p53 as vaccine for therapy of murine tumors. Hughes and Gilleland [*Vaccine* 13:1750 (1995)] used synthetic peptides representing epitopes of outer membrane protein F of *Pseudomonas aeruginosa* to afford protection against *P. aeruginosa* infection in a murine acute pneumonia model. In an attempt to use peptide immunization in humans Brander *et al.* [*Clin Exp Immunol* 105:18 (1996)] showed that a combined

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CD8<sup>+</sup>/CD4<sup>+</sup> T cell-targeted vaccine restimulated the memory CD4<sup>+</sup> T cell response but failed to induce cytotoxic T lymphocytes.

Major sources of exotoxins are, as already mentioned, *S. Aureus* and *S. Pyogenes*. The flesh-eating bacteria, *S. Pyogenes*, produce a family of different toxins with  
5 closely similar mode of action: excessive activation of T cells. *S. Aureus* produces next to SEB as major component, also SEA, SECs, SEE and TSST-1 (toxic shock syndrome toxin 1) and *S. Pyogenes* produces SPE A as major toxin, as well as other pyrogenic exotoxins. Hence, in staphylococcal food poisonings and, more seriously, in biological warfare or in toxic shock caused by *S. pyogenes*, mixtures of toxins are  
10 encountered. The composition of such mixtures cannot be anticipated with certainty. The worst scenarios of biological warfare entail not the use of a single, purified pyrogenic exotoxin, as favored for immunological studies, but rather a readily attainable, crude natural mixtures of such toxins, as produced, for example, by culturing *S. Aureus*.

15 Clearly, this complexity demands the development of broad-spectrum antagonists of pyrogenic exotoxins as well as broad-spectrum vaccines.

There exists, therefore, a long-felt need to design a SEB vaccine that is free of sensitizing potential, yet is capable of protecting test animals or humans against lethal doses of the toxin. Even greater value would be inherent in a vaccine that can  
20 afford protection not only against SEB, but also against a wider spectrum of the SE toxin family, including, for example, SEA.

Moreover, currently, there is no prophylaxis available against SEB or any other pyrogenic exotoxin, nor treatment of exposed persons. There exists, therefore, also a long-felt need to design agents that antagonize the action of SEB, as well as any  
25 other pyrogenic exotoxin. Such antidotes will have great value, both in the medical treatment of acute food poisoning and in saving lives in cases of toxic shock and related pathological conditions.

There exists therefore a need, on the one hand, for an antagonist against pyrogenic exotoxins, for use in immediate treatment, or short term prevention and rapid  
30 prophylaxis, of acute toxic shock and of the harmful effects of such toxins which may be due to, for example, accidental food poisoning, and on the other hand, for a vaccine for immunization against intoxication by pyrogenic exotoxins, for long term protection thereagainst.



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In addition, currently there is no way by which to assess the efficacy of vaccination of humans against pyrogenic toxins, since humans cannot be challenged with the toxin in order to check whether they have been conferred the desired immunity. There exists therefore a need for a clinical test for assessing the efficacy of  
5 vaccination of humans against pyrogenic toxins which employs surrogate markers.

## SUMMARY OF THE INVENTION

The present invention relates to peptides comprising an amino acid sequence substantially homologous to the amino sequence of a fragment of a pyrogenic exotoxin, and derivatives of said peptides, capable of eliciting protective immunity  
10 against toxic shock induced by a pyrogenic exotoxin or by a mixture of pyrogenic exotoxins.

The present invention also relates to peptides comprising an amino acid sequence substantially homologous to the amino sequence of a fragment of a pyrogenic exotoxin, and derivatives of said peptides, capable of antagonizing toxin-mediated  
15 activation of T cells.

The peptides of the invention comprise an amino acid sequence substantially homologous to the amino sequence of a fragment of *Staphylococcal aureus* enterotoxin B (SEB).

The pyrogenic exotoxin is a bacterial exotoxin produced by *Staphylococcus aureus* or *Streptococcus pyogenes*.  
20

According to the invention, the peptides may be further linked through its N-terminus to a lauryl-cysteine (LC) residue and/or through its C-terminus to a cysteine (C) residue, or to other residue/s suitable for linking said peptide to adjuvant/s for immunization.

25 Further, the peptides may be in the form of a dimer, a multimer or in a constrained conformation, in which the constrained conformation is obtained by internal bridges, short-range cyclizations, extension or other chemical modification.

The peptides of the invention are capable of inhibiting expression of pyrogenic toxin-induced mRNA encoded by the IL-2, IFN- $\gamma$  or TNF- $\beta$  genes.

30 In addition, the peptides of the invention are capable of eliciting, in the presence of a suitable immunization adjuvant, in immunized individuals, the production of antibodies that block T-cell pyrogenic toxin-mediated activation.

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In a second aspect of the invention a pharmaceutical composition is provided, for the treatment or short term prophylaxis of toxin-mediated activation of T cells, comprising as active ingredient a therapeutically effective amount of at least one peptide according to the invention or derivative thereof.

- 5    Additionally, the invention provides a vaccine for conferring long term immunity against toxic shock induced by at least one pyrogenic exotoxin, comprising as active ingredient an immunologically effective amount of at least one peptide according to the invention, or derivative thereof.

- 10   Also provided by the invention is a method for the immediate treatment or short term prevention of acute toxic shock and of the harmful effects which may be due to, for example, accidental food poisoning induced by at least one pyrogenic exotoxin, comprising administering to a patient in need of such treatment a therapeutically effective amount of the pharmaceutical composition of the invention or of a therapeutically effective amount of at least one peptide of the invention.

- 15   Additionally, the invention provides a method for conferring long term immunity to toxic shock induced by a pyrogenic exotoxin or a mixture of pyrogenic exotoxins, comprising administering to a patient an effective immunizing amount of a vaccine of the invention.

- 20   In yet a further aspect, the invention relates to an antiserum containing antibodies directed against at least one peptide of the invention, which peptide can elicit the production of the antibodies. The antiserum is capable of alleviating toxic shock induced by a pyrogenic exotoxin.

- 25   In addition, the invention provides for a method for assessing the efficacy of a vaccine for conferring immunity against one or more pyrogenic toxins comprising determining the ability of serum from an immunized individual to antagonize toxin-mediated activation of T cells. A kit for assessing the efficacy of a vaccine for conferring immunity against one or more pyrogenic toxins comprising determining the ability of serum from an immunized individual to antagonize toxin-mediated activation of T cells by the method of the invention is also provided.

### 30   **DESCRIPTION OF THE FIGURES**

**Figure 1A-1E**    *Induction of IL-2, IFN- $\gamma$  and TNF- $\beta$  Gene Expression by SEB*  
Aliquots of  $4 \times 10^6$  human PBMC were induced with SEB. Total RNA was extracted at times indicated [T(h)] and serial twofold

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dilutions (vertical rows) were subjected to dot blot hybridization analysis with  $^{32}\text{P}$ -labeled IL-2 and IFN- $\gamma$  anti-sense RNA probes; autoradiograms (Figs. 1B, 1C) were quantitated by densitometry at 630 nm, plotted in (Fig. 1A). In separate experiments, aliquots of  $3 \times 10^7$  human PBMC were induced with SEB and total RNA was extracted at times indicated. IL-2, IFN- $\gamma$  (Fig. 1D) and TNF- $\beta$  mRNA (Fig. 1E) was quantitated by RNase protection analysis. IL-2 mRNA protects a fragment of 117 nt; IFN- $\gamma$  mRNA protects a fragment of 183 nt; TNF- $\beta$  mRNA protects 2 fragments of 274 and 263 nt.  $\beta$ -Actin RNA served as loading control.

**Figure 2***Secondary structure domains within the SEB protein molecule*

The two structural domains of the molecule are seen clearly. Cylinders and arrows represent  $\alpha$ -helices and  $\beta$ -strands, respectively. The residues forming the secondary structural elements are: 13-17 ( $\alpha 1$ ), 21-29 ( $\alpha 2$ ), 33-39 ( $\beta 1$ ), 48-52 ( $\beta 2$ ), 63-68 ( $\beta 3$ ), 70-78 ( $\alpha 3$ ), 81-89 ( $\beta 4$ ), 112-120 ( $\beta 5$ ), 127-138 ( $\beta 6$ ), 141-151 ( $\beta 7$ ), 154-156 ( $\beta 8$ ), 157-172 ( $\alpha 4$ ), 182-190 ( $\beta 9$ ), 195-200 ( $\beta 10$ ), 210-217 ( $\alpha 5$ ), 222-224 ( $\beta 11$ ), and 229-236 ( $\beta 12$ ). Black domain covers pSEB(150-161) and p12(150-161) (see Table 1). Adapted from Swaminathan *et al.* [Swaminathan *et al.*, Nature **359**:801 (1992)].

**Figure 3***Lack of SEB agonist activity of SEB-derived peptides*

Aliquots of  $4 \times 10^6$  PBMC were induced without inducer (-SEB), with SEB (+SEB), or with 1  $\mu\text{g}/\text{ml}$  of one of the indicated SEB-related peptides (Table 1) as sole inducer. Total RNA was extracted after 4 h of induction and serial twofold dilutions were subjected to dot blot hybridization analysis with  $^{32}\text{P}$ -labeled IL-2 and IFN- $\gamma$  anti-sense RNA probes. Autoradiograms were subjected to microdensitometry at 630 nm;  $A_{630}$  is plotted.

**Figure 4A-4B***Antagonist activity of SEB-related peptides*

Aliquots of  $4 \times 10^6$  PBMC were induced without inducer (-SEB), with SEB in the presence of no peptide (NP), or with SEB in the presence of 1  $\mu\text{g}/\text{ml}$  of one of the indicated SEB-related peptides (sequences: Table 1). Total RNA was extracted after 4 hrs of induction and serial twofold dilutions (vertical rows) were subjected to dot blot hybridization analysis with  $^{32}\text{P}$ -labeled IL-2

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and IFN- $\gamma$  anti-sense RNA probes. Autoradiograms shown were quantitated by densitometry at 630 nm, plotted in (Fig. 4A). Fold inhibition (f.i.) of IL-2 and IFN- $\gamma$  mRNA expression induced by SEB (Fig. 4B) was calculated from data as shown in (Fig. 4A), by subtracting basal levels of mRNA expressed (-SEB) and dividing extent of mRNA expression induced by SEB alone (NP) by that obtained with SEB in the presence of the indicated SEB-related peptide.

**Figure 5** *Inhibition of SEB-mediated induction of IL-2 and IFN- $\gamma$  mRNA by p12(150-161)*

Aliquots of  $3 \times 10^7$  PBMC were induced with SEB, SEB-related peptides as indicated, or both. At times shown, total RNA was extracted and subjected to RNase protection analysis, using a  $^{32}\text{P}$ -labeled IL-2 or IFN- $\gamma$  antisense RNA probe as for Fig. 1D. Autoradiograms are shown. Data for IL-2 and IFN- $\gamma$  are derived from separate experiments.

**Figure 6** *Inhibition of SEB-mediated induction of IL-2, IFN- $\gamma$  and TNF- $\beta$  mRNA by p12(150-161)*

Aliquots of  $3 \times 10^7$  PBMC were induced with SEB, in the presence of no peptide (NP) or of SEB-related peptides as indicated. At times shown, total RNA was extracted and subjected to RNase protection analysis, using a  $^{32}\text{P}$ -labeled IL-2, IFN- $\gamma$  or TNF- $\beta$  antisense RNA probe as for Figs. 1D and 1E. rRNA served as loading control. Autoradiograms are shown.

**Figure 7A-7B** *SEB antagonist activity of p12(150-161) monomer, dimer and trimer*

(Fig. 7A) Aliquots of  $4 \times 10^6$  PBMC were induced with 100 ng/ml of SEB alone ( $\circ$ ), or with SEB in the presence of p12(150-161) monomer ( $\bullet$ ), dimer ( $\blacksquare$ ) or trimer ( $\blacktriangle$ ); molar excess of each peptide over SEB was 420-fold. (Fig. 7B) Dose response to dimer in the same experiment. Dimer was used at a concentration as in (Fig. 7A) ( $\blacksquare$ ) or diluted 1:10 ( $\blacksquare$ ) or 1:100 ( $\blacksquare$ ). At times indicated [T(h)], total RNA was extracted and serial twofold dilutions were subjected to dot blot hybridization analysis with  $^{32}\text{P}$ -labeled IFN- $\gamma$  antisense RNA probe. Autoradiograms were subjected to micro-densitometry at 630 nm;  $A_{630}$  is plotted.

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- Figure 8A-8D** *SEB antagonist activity of Cys-p12(150-161)*  
 Aliquots of  $4 \times 10^6$  PBMC were induced with 100 ng/ml of SEB alone ( $\circ$ ), or with SEB in the presence of undiluted peptide ( $\blacktriangle$ ) (in 420-fold molar excess over SEB), or of peptide diluted 1:10 ( $\bullet$ ) or 1:100 ( $\blacksquare$ ). In Figs. 8A and 8C, p12(150-161) was used. In Figs. 8B and 8D, Cys-p12(150-161) was used, which is p12(150-161) carrying a Cys residue at both N- and C-termini. At times indicated [T(h)], total RNA was extracted and serial twofold dilutions were subjected to dot blot hybridization analysis with  $^{32}\text{P}$ -labeled IL-2 (Figs. 8A and 8B), or IFN- $\gamma$  (Figs. 8C and 8D) antisense RNA probe. Autoradiograms were subjected to microdensitometry at 630 nm;  $A_{630}$  is plotted.
- Figure 9** *Lack of antagonist activity of pSEB(150-161) for TSST-1*  
 Aliquots of  $3 \times 10^7$  PBMC were induced with SEB or TSST-1 as shown, in the presence of no peptide (NP) or of 1  $\mu\text{g}/\text{ml}$  of pSEB(41-61) or pSEB(150-161) as indicated. At times shown (h), total RNA was extracted and subjected to RNase protection analysis, using a  $^{32}\text{P}$ -labeled IL-2 antisense RNA probe as for Fig. 1D.
- Figure 10A-10D** *Lack of antagonist activity of p12(150-161) for SEA*  
 Aliquots of  $4 \times 10^6$  human PBMC were induced with SEB (Figs. 10A, 10B) or SEA (Figs. 10C, 10D), in the absence ( $\circ$ ,  $\Delta$ ) or presence ( $\bullet$ ,  $\blacktriangle$ ) of 1  $\mu\text{g}/\text{ml}$  of peptide p12(150-161). Total RNA was extracted at times [T(h)] indicated and serial twofold dilutions were subjected to dot blot hybridization analysis with  $^{32}\text{P}$ -labeled IL-2 (Figs. 10A, 10C) and IFN- $\gamma$  (Figs. 10B, 10D) anti-sense RNA probes. Autoradiograms were quantitated by densitometry at 630 nm.
- Figure 11** *Toxin antagonist activity of p12(150-161) dimer and D-Ala forms: SEB and TSST-1*  
 Aliquots of  $3 \times 10^7$  PBMC were induced with SEB or TSST-1 as indicated, in the presence of no peptide (NP) or of 10  $\mu\text{g}/\text{ml}$  of p12(150-161) or, where indicated, an equal molar concentration of p12(150-161) dimer (dimer) or of p12(150-161) carrying a D-Ala residue at both N- and C-termini (D-Ala). At times shown, total RNA was extracted and subjected to RNase protection

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analysis, using  $^{32}\text{P}$ -labeled IL-2 and IFN- $\gamma$  antisense RNA probes as for Figure 1D. rRNA served as loading control.

**Figure 12**

*Toxin antagonist activity of p12(150-161) dimer and D-Ala forms: SEB, TSST-1 and SEA*

5 Aliquots of  $3 \times 10^7$  PBMC were induced with SEB, TSST-1 or SEA as indicated, in the presence of no peptide (NP), p12(150-161) dimer (**dimer**) or p12(150-161) carrying a D-Ala residue at both N- and C-termini (**D-Ala**) as indicated, each of the latter at a molar concentration equivalent to 10  $\mu\text{g/ml}$  of p12(150-161). At  
10 times shown, total RNA was extracted and subjected to RNase protection analysis, using  $^{32}\text{P}$ -labeled IL-2 antisense RNA probe as for Fig. 1D. rRNA served as loading control.

**Figure 13A-13B** *Effect of anti-SEB peptide sera on induction of IL-2 and IFN- $\gamma$  mRNA by SEB*

15 Aliquots of  $4 \times 10^6$  PBMC were induced with SEB (**control, C**) ( $\bullet$ ). Rabbit sera against SEB peptides p12LC(150-161) ( $\blacktriangle$ ), pSEBLC(13-33) ( $\circ$ ), pSEB(81-93) ( $\square$ ) or pSEB(41-61) ( $\blacksquare$ ) in 1:100 dilution were included from the onset of induction. At times [**T(h)**] indicated, total RNA was extracted and serial  
20 twofold dilutions (vertical rows) were subjected to dot blot hybridization analysis with  $^{32}\text{P}$ -labeled IL-2 (Fig. 13A) and IFN- $\gamma$  (Fig. 13B) anti-sense RNA probes. Only autoradiograms for IL-2 are shown. Autoradiograms were quantitated by densitometry at 630 nm (Figs. 13A, 13B).

**Figure 14A-14B** *Effect of anti-SEB peptide sera on induction of IL-2 and IFN- $\gamma$  mRNA by SEA*

25 Aliquots of  $4 \times 10^6$  PBMC were induced with SEA (**control, C**). Where shown, rabbit sera against SEB peptides p12LC(150-161), pSEBLC(13-33), pSEB(81-93) or pSEB(41-61) in 1:100 dilution  
30 were included from the onset of induction. At 6 hrs (Fig. 14A) and 20 hrs (Fig. 14B), total RNA was extracted and serial twofold dilutions (vertical rows) were subjected to dot blot hybridization analysis with  $^{32}\text{P}$ -labeled IL-2 (Fig. 14A) and IFN- $\gamma$  (Fig. 14B) anti-sense RNA probes; autoradiograms shown were  
35 quantitated by densitometry at 630 nm.

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**Figure 15**      *Effect of anti-SEB peptide sera on induction of IL-2 and IFN- $\gamma$  mRNA by TSST-1*

5      Aliquots of  $3 \times 10^7$  PBMC were induced with 100 ng/ml of TSST-1 (control, (C)). Where shown, rabbit sera against SEB peptides p12LC(150-161) or pSEBLC(13-33) in  $1:10^4$  dilution were included from the onset of induction. At times shown, total RNA was extracted and subjected to RNase protection analysis, using  $^{32}\text{P}$ -labeled IL-2 and IFN- $\gamma$  antisense RNA probes as for Figure 1D. rRNA served as loading control.

10      **DETAILED DESCRIPTION OF THE INVENTION**

In search for efficient agents for long and short term treatment and prophylaxis of toxic shock induced by pyrogenic exotoxins, a series of short peptides related to certain domains within the SEB protein molecule has been designed. The biological properties of different synthetic SEB-related peptides were then evaluated by the  
15      following several criteria:

1. Lack of SEB agonist activity, assayed by the ability to induce in peripheral blood mononuclear cells from normal human blood donors (PBMC) the expression of mRNA encoded by the IL-2 and IFN- $\gamma$  genes, in the absence of any other inducing agent;
- 20      2. pyrogenic exotoxin antagonist activity, assayed by the ability to inhibit, in PBMC, the expression of mRNA encoded by the IL-2, IFN- $\gamma$ , and TNF- $\beta$  genes induced by a pyrogenic exotoxin such as SEB;
3. Immunogenicity, assayed by the ability of a peptide to elicit, in immunized rabbits, the production of immunoglobulin G (IgG) antibodies that bind SEB;
- 25      4. Immunogenicity, assayed by the ability of a peptide to elicit, in immunized rabbits, the production of antibodies that block the harmful action of pyrogenic exotoxins, such as SEB on the human cellular immune response, monitored by the ability of rabbit serum raised against a peptide to inhibit, in PBMC, the induction of IL-2 and IFN- $\gamma$  mRNA by SEB or by other pyrogenic exotoxins  
30      such as TSST-1 or the more related toxin, SEA;
5. Activity of a vaccine, such as anti-SEB vaccine, assayed by the ability of a peptide to protect immunized animals, in the D-galactosamine mouse model,

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against lethal doses of SEB whether administered via the intramuscular or intranasal route.

- The inventors have indeed obtained peptides that meet each of these five criteria. In a specific embodiment of the invention, a SEB-related dodecapeptide antagonist was designed. This peptide blocks the action of SEB as well as other pyrogenic exotoxins on the human immune response *in vitro*, severely inhibiting SEB-mediated induction of IL-2, IFN- $\gamma$  and TNF- $\beta$  mRNA. It is clear that this peptide could be used for treatment of acute toxic shock and of harmful effects which may be due to, for example, accidental food poisoning induced by pyrogenic exotoxins.
- 10 In addition, this peptide elicits antibodies that protect human lymphoid cells against SEB, SEA, and TSST-1, indicating that it may confer wider protective immunity against pyrogenic toxins. However, antibodies raised against peptides derived from certain other SEB protein domains actually enhanced the response of human PBMC to SEB and SEA, as expressed by greater induction of IL-2 and IFN- $\gamma$  mRNA, rather than protecting against the toxins. Immunization of mice with the SEB antagonist peptide elicited protection against lethal doses of SEB, resulting in survival of test animals. It is clear that the peptide may also be used for conferring long-term immunity against toxic shock induced by pyrogenic exotoxins.

- Thus, in a first aspect, the present invention relates to peptides comprising an amino acid sequence substantially homologous to the amino sequence of a fragment of a pyrogenic exotoxin, and to functional derivatives of such peptides, capable of eliciting protective immunity against toxic shock induced by the exotoxins. The terms derivatives and functional derivatives used herein mean peptides with any insertions, deletions, substitutions and modifications that are capable of eliciting protective immunity against toxic shock induced by the exotoxins and/or of antagonizing toxin-mediated activation of T cells (hereafter referred to as "derivative/s").

- In a second aspect the invention relates to peptides comprising an amino acid sequence substantially homologous to the amino sequence of a fragment of a pyrogenic exotoxin, and to derivatives of such peptides, capable of antagonizing toxin-mediated activation of T cells.

The pyrogenic exotoxin is usually a bacterial exotoxin, specifically an exotoxin produced by *Staphylococcus aureus* or by *Streptococcus pyogenes*.



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In a preferred embodiment of both said aspects of the invention, the invention relates to a peptide comprising an amino acid sequence substantially homologous to the amino sequence of a fragment of *Staphylococcal aureus* enterotoxin B (SEB).

5 In a specifically preferred embodiment the invention relates to peptides comprising the amino acid sequence shown in SEQ ID NO:1 (positions 150 to 161 of the sequence of the naturally occurring SEB protein shown in SEQ ID NO:12), and functional derivatives thereof, capable of eliciting protective immunity against toxic shock induced by at least one pyrogenic exotoxin and/or of antagonizing toxin-mediated activation of T cells. These peptides can therefore be used for both  
10 immediate treatment of acute toxic shock and of the harmful effects which may be due to, for example, accidental food poisoning, induced by the pyrogenic exotoxins and for conferring long-term immunity against such toxic shock.

A particular example may be a peptide having the amino acid sequence shown in SEQ ID NO:1 (hereafter also referred to as pSEB(150-161)) and functional  
15 derivatives thereof. This peptide is capable of eliciting protective immunity against toxic shock induced by at least one pyrogenic exotoxin and/or of antagonizing toxin-mediated activation of T cells.

In an alternative embodiment the invention relates to peptides comprising the amino acid sequence shown in SEQ ID NO:2 and to derivatives thereof, capable of  
20 eliciting protective immunity against toxic shock induced by at least one pyrogenic exotoxin and/or of antagonizing toxin-mediated activation of T cells. Also these peptides can be used for both immediate treatment of acute toxic shock and of the harmful effects which may be due to, for example, accidental food poisoning induced by the pyrogenic exotoxins and for conferring long-term immunity against  
25 such toxic shock. A particular example may be a peptide having the amino acid sequence shown in SEQ ID NO:2 (hereinafter also referred to as p12(150-161)) and derivatives thereof capable of eliciting protective immunity against toxic shock induced by at least one pyrogenic exotoxin and/or of antagonizing toxin-mediated activation of T cells.

30 In a further embodiment the invention relates to peptides comprising the amino acid sequence shown in SEQ ID NO:3 (positions 152 to 161 of the sequence of the naturally occurring protein shown in SEQ ID NO:12) and to functional derivatives thereof, capable of eliciting protective immunity against toxic shock induced by at least one pyrogenic exotoxin and/or of antagonizing toxin-mediated activation of T  
35 cells. Also these peptides can be used for both immediate treatment of acute toxic

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shock and of the harmful effects which may be due to, for example, accidental food poisoning induced by the pyrogenic exotoxins and for conferring long-term immunity against such toxic shock.

5 Particular examples may be a peptide having the amino acid sequence shown in SEQ ID NO:3 (hereinafter also referred to as pSEB(152-161)) and derivatives thereof capable of eliciting protective immunity against toxic shock induced by a pyrogenic exotoxin or a mixture of pyrogenic exotoxins and/or of antagonizing toxin-mediated activation of T cells.

10 In addition, the invention relates to peptides comprising the amino acid sequence shown in SEQ ID NO:4 and to functional derivatives thereof, capable of eliciting protective immunity against toxic shock induced by at least one pyrogenic exotoxin and/or of antagonizing toxin-mediated activation of T cells. Also these peptides can be used for both immediate treatment of acute toxic shock and of the harmful effects which may be due to, for example, accidental food poisoning induced by the  
15 pyrogenic exotoxins and for conferring long-term immunity against such toxic shock.

A particular example may be a peptide having the amino acid sequence shown in SEQ ID NO:4 (hereinafter also referred to as p10(152-161)) and functional derivatives thereof, capable of eliciting protective immunity against toxic shock  
20 induced by a pyrogenic exotoxin or a mixture of pyrogenic exotoxins and/or of antagonizing toxin-mediated activation of T cells.

As mentioned, pSEB(150-161) corresponds to the natural amino acid sequence in SEB between positions 150-161, while p12(150-161) differs from the corresponding natural amino acid sequence of SEB in 3 out of the 12 positions, yet possesses even  
25 greater toxin antagonist activity. It is thus possible to design a pyrogenic exotoxin antagonist and/or vaccine through use of short peptides related, but not necessarily identical to, domains within the SEB toxin molecule.

The lack of structure of linear peptides on one hand renders them vulnerable to proteases in human serum and on the other hand acts to reduce their affinity for  
30 target sites, because only few of the possible conformations may be active. Therefore, it is desirable to optimize antagonist peptide structure.

Thus, the peptides of the invention can be coupled through their N-terminus to a lauryl-cysteine (LC) residue and/or through their C-terminus to a cysteine (C)

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residue, or to other residue/s suitable for linking the peptide to adjuvant/s for immunization, as will be described in more detail hereafter.

Accordingly, in a further embodiment, the invention relates to a peptide having the amino acid sequence shown in SEQ ID NO:5 (hereinafter also referred to as  
5 pSEBLC(150-161)) and functional derivatives thereof, capable of eliciting protective immunity against toxic shock induced by pyrogenic exotoxins and/or of antagonizing toxin-mediated activation of T cells. These peptides can also be used for both immediate treatment of acute toxic shock and of the harmful effects which may be due to, for example, accidental food poisoning induced by the pyrogenic  
10 exotoxins and for conferring long-term immunity against such toxic shock.

In yet another embodiment, the invention relates to a peptide having the following amino acid sequence shown in SEQ ID NO:6 (hereinafter also referred to as  
15 p12LC(150-161)) and functional derivatives thereof capable of eliciting protective immunity against toxic shock induced by pyrogenic exotoxins and/or of antagonizing toxin-mediated activation of T cells. Evidently, such peptides can also be used for both immediate treatment of acute toxic shock and of the harmful effects which may be due to, for example, accidental food poisoning induced by the pyrogenic exotoxins and for conferring long-term immunity against such toxic shock.

20 The peptides of the invention, as well as the derivatives thereof may all be positively charged, negatively charged or neutral and may be in the form of a dimer, a multimer or in a constrained conformation.

A constrained conformation can be attained by internal bridges, short-range cyclizations, extension or other chemical modification.

25 Peptides in the form of a dimer or trimer can have, for example, the amino acid sequences shown in SEQ ID NOs. 7 and 8, respectively (hereinafter also referred to as Dimer and Trimer, receptively) and functional derivatives thereof, capable of eliciting protective immunity against toxic shock induced by pyrogenic exotoxins and/or of antagonizing toxin-mediated activation of T cells. As with other peptides  
30 of the invention, these peptides can also be used for both immediate treatment of acute toxic shock and of the harmful effects caused thereby and for conferring long-term immunity against such toxic shock.

Further, the peptides of the invention may be extended at the N-terminus and/or C-terminus thereof with various identical or different amino acid residues. As an

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example for such extension, the peptide may be extended at the N-terminus and/or C-terminus thereof with identical or different hydrophobic amino acid residue/s which may be naturally occurring or synthetic amino acid residue/s. A preferred synthetic amino acid residue is *D*-alanine.

- 5 A particular example for a peptide extended with synthetic amino acid residues is the peptide having the amino acid sequence shown in SEQ ID NO:10 (hereinafter also referred to as *D*-Ala) and functional derivatives thereof, capable of eliciting protective immunity against toxic shock induced by pyrogenic exotoxins and/or of antagonizing toxin-mediated activation of T cells.
- 10 An additional example for such an extension is provided by peptides extended both at the *N*-terminus and/or *C*-terminus thereof with a cysteine residue. Naturally, such an extension may lead to a constrained conformation due to Cys-Cys cyclization resulting from the formation of a disulfide bond. A particular such peptide has the amino acid sequence shown in SEQ ID NO:9 (hereinafter also referred to as Cys-
- 15 p12(150-162)) and functional derivatives thereof, capable of eliciting protective immunity against toxic shock induced by pyrogenic exotoxins and/or of antagonizing toxin-mediated activation of T cells. According to one embodiment of the invention, the Cys-p12(150-162)) is a cyclic peptide having a disulfide bond via the terminal cysteine residues. Nevertheless, the Cys-p12(150-162) peptide may be
- 20 linear.

- In addition the peptide may be extended aromatic amino acid residue/s, which may be naturally occurring or synthetic amino acid residue/s. A preferred aromatic amino acid residue is tryptophan. Alternatively, the peptides can be extended at the N-terminus and/or C-terminus thereof with amino acids present in corresponding
- 25 positions of the amino acid sequence of the naturally occurring pyrogenic exotoxin.

- Nonetheless, according to the invention, the peptides of the invention may be extended at the *N*-terminus and/or *C*-terminus thereof with various identical or different organic moieties which are not a naturally occurring or synthetic amino acids. As an example for such extension, the peptide may be extended at the N-
- 30 terminus and/or *C*-terminus thereof with an *N*-acetyl group. A particular example for such an extension is the peptide having the amino acid sequence shown in SEQ ID NO:11 (hereinafter also referred to as Ac-p12(150-161)) and functional derivatives thereof, capable of eliciting protective immunity against toxic shock induced by pyrogenic exotoxins and/or of antagonizing toxin-mediated activation of
- 35 T cells.

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These extended peptides, as other peptides of the invention, can also be used for both immediate treatment of acute toxic shock and of the harmful effects caused thereby and for conferring long-term immunity against such toxic shock.

5 The peptides of the invention are capable of inhibiting expression of pyrogenic toxin-induced mRNA encoded by the IL-2, IFN- $\gamma$  or TNF- $\beta$  genes, as will be shown in the following Examples.

In addition, the peptides of the invention are capable of eliciting the production of antibodies that block T-cell activation in immunized individuals. The production of antibodies will be enhanced in the presence of a suitable immunization adjuvant.  
10 Preferred adjuvants may be keyhole limpet hemocyanin (KLH), proteosomes or alum.

As will be shown in the Examples, peptide p12(150-161) and the specific derivatives thereof, i.e. the Dimer form, the Cys form (Cys-p12(150-161)) and D-Ala form exhibit antagonist activity against SEB as well as against other pyrogenic  
15 exotoxins. The amino acid sequence of these synthetic peptides having the sequence shown in SEQ ID NOs.:2, 7, 9 and 10, differ in several positions from the corresponding sequence in SEB, T N K K K V T A Q E L D, found in peptide pSEB(150-161). The K K K and Q E L D motifs, features shared by peptides p12(150-161) (and its derivatives) and pSEB(150-161), are spaced equally in both  
20 and may be important for antagonist activity, with the triple-lysine motif K K K conferring 3 positive charges. Residues T150, K152, E159 and D161 of this SEB domain are conserved among all staphylococcal enterotoxins [Swaminathan *et al.* (1992) *ibid.*]. Indeed, the 150-161 domain of SEB is highly conserved among pyrogenic toxins in general, with 10/12 identities for SEA, SEC1, SEC2, and *S. pyogenes* exotoxin A (SPE A) and 9/12 for SEE [Bohach and Schlievert, Mol Genet  
25 209:5 (1987); Couch *et al.*, J Bacteriol 170:2954 (1988); Bohach and Schlievert, Infect Immun 57:2249 (1989)]. All of these toxins contain the residues underlined above, including the first 2 lysine residues (K K) and the Q E L D [Swaminathan *et al.* (1992) *ibid.*; Bohach and Schlievert (1987) *ibid.*; Couch *et al.* (1988) *ibid.*; Bohach and Schlievert (1989) *ibid.*]. The superantigen, pep M5 protein, also  
30 contains a region with limited homology (<50%) to pSEB(150-161) located near its C-terminus [Wang *et al.*, J Immunol 151:1419 (1993)].

Conservation of SEB domains among the pyrogenic toxin family is, however, not unique for amino acids 150-161. SEB domains covering amino acids 76-86, 113-  
35 124, 151-168 and 213-226 all show extensive conservation within this family

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[Hoffmann *et al.*, Infect Immun 62:3396 (1994)]. Moreover, the relevance of the conservation of the above-mentioned motifs corresponding to part of the sequence in pSEB(150-161) is not clear, since this region does not contribute to mitogenicity [Wang *et al.* (1993) *ibid.*].

- 5 In a further aspect, the invention relates to pharmaceutical compositions for the treatment or prophylaxis of toxin-mediated activation of T cells, comprising as active ingredient a therapeutically effective amount of at least one peptide according to the invention or derivative thereof. As described above, the peptides comprise an amino acid sequence substantially homologous to the amino acid sequence of a  
10 fragment SEB.

The term toxin-mediated activation as used throughout this application can mean activation of T cells mediated by a single pyrogenic exotoxin or a mixture of such toxins.

- 15 The pharmaceutically 'effective amount' for purposes herein is that determined by such considerations as are known in the art. The amount must be sufficient to antagonize toxin-mediated activation of T cells.

- The pharmaceutical composition of the invention can be prepared in dosage units forms and may be prepared by any of the methods well-known in the art of pharmacy. In addition, the pharmaceutical compositions of the invention may  
20 further comprise pharmaceutically acceptable additives such as pharmaceutical acceptable carrier, excipient or stabilizer, and optionally other therapeutic constituents. Naturally, the acceptable carriers, excipients or stabilizers are non-toxic to recipients at the dosages and concentrations employed.

- 25 The magnitude of therapeutic dose of the composition of the invention will of course vary with the group of patients (age, sex, etc.), the nature of the condition to be treated and with the route administration and will be determined by the attending physician.

- 30 In yet a further embodiment, the invention relates to vaccines for conferring immunity against toxic shock induced by pyrogenic exotoxins, comprising as active ingredient an immunologically effective amount of at least one peptide according to the invention or derivatives thereof and may contain mixtures of such peptides and derivatives.

By the term 'immunologically effective amount' is meant any amount sufficient to enhance the production of antibodies that block T cell activation induced by

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pyrogenic exotoxins, and confer immunity against toxic shock induced by a pyrogenic exotoxin or by a mixture of pyrogenic exotoxins.

The vaccines of the invention may optionally further comprise suitable immunization adjuvants or mixtures thereof. Suitable adjuvants may be  
5 proteosomes, KLH and alum, as well as combinations of proteosomes with alum and of KLH with alum.

As will be shown in the following Examples, the vaccines according to the invention are capable of enhancing production of antibodies that block T-cell activation induced by pyrogenic exotoxins.

10 Efforts to develop antidotes for use against toxic shock symptoms have concentrated on blocking downstream phenomena in the toxicity cascade, mainly by inhibiting the action of TNF with monoclonal antibodies or soluble receptors. The high levels of cytokines produced in response to toxins render this approach ineffective. The present Invention shows that it is possible to block the action of a pyrogenic  
15 exotoxin by an altogether different strategy, using antagonists that inhibit toxin action at the top of the toxicity cascade, before activation of T cells takes place.

The Examples describe in detail molecular methods, analysis of pyrogenic exotoxin-mediated activation of the human cellular immune response through expression of IL-2, IFN- $\gamma$ , and TNF- $\beta$  genes in PBMC, to evaluate toxin antagonist activity.  
20 Studies in human PBMC were combined with animal tests to evaluate immunogenic properties and vaccine efficacy and it will be shown that these methods are applicable in devising agents that counteract or protect human PBMC also against other members of the family of pyrogenic exotoxins.

Because humans are far more sensitive to pyrogenic exotoxins than mice, while  
25 primate models poses other limitations such as cost, there is a need for a human *in vitro* system, capable of analyzing the mechanisms of toxin-mediated activation and suppression of the immune response. The present invention provides such a system, which offers major advantages:

a) The experimental system employs freshly prepared human lymphoid cell  
30 populations that preserve cell-cell interactions involved in regulation of cytokine production and are as close as possible to the peripheral immune system of the body;

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- b) Early events of the immune response can be analyzed precisely and directly by following the transient and highly regulated expression of IL-2, IFN- $\gamma$  and TNF- $\beta$  mRNA;
- c) Expression of IL-2, IFN- $\gamma$  and TNF- $\beta$  genes is exquisitely sensitive to activation elicited by SEB;
- d) This molecular approach is far more direct and specific than measurement of biological responses, such as cell proliferation or antibody production, that are the cumulative result of a sequence of events;
- e) The approach presented offers a tool for mapping functional domains in SEB essential for activation of human IL-2, IFN- $\gamma$  and TNF- $\beta$  genes, and can serve to facilitate both antagonist and vaccine development.

Thus, the invention also relates to a method for treating toxic shock induced by a pyrogenic exotoxin or a mixture of pyrogenic exotoxins. The method comprises administering to a patient in need of such treatment a therapeutically effective amount of the pharmaceutical composition of the invention or a therapeutically effective amount of at least one peptide of the invention, or functional derivative thereof.

In a further embodiment there is provided a method for preventing toxic shock induced by a pyrogenic exotoxin or a mixture of pyrogenic exotoxins comprising administering to a patient in need of such treatment a therapeutically effective amount of a composition of the invention or of a therapeutically effective amount of at least one peptide of the invention or functional derivative thereof.

The invention also relates to a method for immunizing a patient against toxic shock induced by pyrogenic exotoxin, comprising administering to a patient an effective immunizing amount of the vaccine of the invention or of at least one peptide of the invention or functional derivative thereof.

The magnitude of therapeutic dose of the peptide or of the vaccine of the invention will of course vary with the group of patients (age, sex etc.), the nature of the condition to be treated and with the route administration and will be determined by the attending physician.

The peptides and the vaccines of the invention can be administered and dosed in accordance with good medical practice. In particular, the immunization method of the invention comprises a single administration of the peptides or vaccines of the invention. Administration may be carried out in various ways, including



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intravenous, intramuscular or subcutaneous injection. However, other methods of administration such as intranasal administration are also possible.

As shown hereafter, antibodies raised against peptides derived from certain SEB protein domains actually enhance the response of human PBMC to SEB and SEA, as expressed by induction of IL-2 and IFN- $\gamma$  mRNA, rather than protect against these toxins. This would provide a tool for detecting such potential exacerbation by any pyrogenic exotoxin vaccine even before trials with humans are conducted. Design of pyrogenic exotoxin antagonist peptides as described herein may find novel applications not only in hitherto neglected areas, prophylaxis against pyrogenic exotoxins and treatment of toxin-exposed individuals but also may facilitate the development of a safer pyrogenic exotoxin vaccine. A defined peptide vaccine, free of exacerbating properties, would be superior to a toxoid vaccine.

Thus, the invention also relates to antisera containing antibodies directed against peptides of the invention. For example, peptides such as pSEBLC(150-161) or p12LC(150-161) can be linked through the lauryl cysteine residue to proteosomes. Alternatively, peptides such as those coupled through their C-terminus to a cysteine (C) residue, as described above, can be linked through the cysteine residue to KLH. KLH and proteosomes are known adjuvants for immunization and the peptides so linked are capable of eliciting the production of antibodies.

It is also known in the art that aluminum hydroxide (alum) may be used as an immunization adjuvant directly, with non-linked peptides, or after linking a peptide to proteosomes or to KLH [Lowell *et al.*, (1996) *ibid.*] or other suitable adjuvants. Therefore, the invention also relates to antisera containing antibodies directed against peptides of the invention, or functional derivatives thereof. The antisera of the invention are capable of alleviating toxic shock induced by a pyrogenic exotoxin. An antiserum according to the invention can be a domestic animal antiserum, for example rabbit, sheep, bovine, equine, porcine or goat antiserum.

For some purposes, for example the *in vitro* use mentioned above, human sera from immunized individuals can be used.

As already mentioned above, at present there exists no tool for assessing the efficacy of a vaccine against pyrogenic toxins in conferring immunity thereagainst. The present invention provides for such a method and thus, in a further aspect the invention relates to a method for assessing the efficacy of a vaccine for conferring immunity against one or more pyrogenic toxins comprising determining the ability

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of serum from an immunized individual to antagonize toxin-mediated activation of T cells.

The ability of serum from an immunized individual to antagonize toxin-mediated activation of T cells can be determined by measuring the inhibition of expression of pyrogenic toxin-induced mRNA encoded by the IL-2, IFN- $\gamma$ , or TNF- $\beta$  genes. Reference may be made to Example 7 and Fig. 13 to 15.

A kit for assessing the efficacy of a vaccine for conferring immunity against one or more pyrogenic toxins comprising determining the ability of serum from an immunized individual to antagonize toxin-mediated activation of T cells by the method of the invention is also provided.

## EXAMPLES

### *Cell culture and induction of human cytokine gene expression*

PBMC from healthy human donors were separated on Ficoll Paque (Pharmacia), washed twice with 50 ml of RPMI 1640 medium, resuspended at a density of  $4 \times 10^6$ /ml and cultured in this medium supplemented with 2% fetal calf serum, 2 mM glutamine, 10 mM MEM nonspecific amino acids, 100 mM Na-pyruvate, 10 mM Hepes pH 7.2,  $5 \times 10^{-5}$  M 2-mercapto-ethanol, 100 u/ml penicillin, 100  $\mu$ g/ml streptomycin and 5  $\mu$ g/ml nystatin. SEB (lot 14-30, from the Department of Toxinology, U.S. Army Medical Research Institute of Infectious Diseases), SEA or TSST-1 (Sigma) were added to 100 ng/ml.

### *RNase protection analysis*

Total RNA was extracted with guanidinium isothiocyanate [Chomczynski and Sacchi, Anal Biochem **162**:156 (1987)]. RNase protection analysis was done [Arad *et al.* (1995) *ibid.*] using genomic antisense RNA probes transcribed with [ $\alpha$ - $^{32}$ P]UTP *in vitro* from DNA inserted into pBS (Promega). The IL-2 probe (600 nucleotides (nt)), transcribed from the T7 promoter, is complementary to the third exon and a portion of the third intron of the IL-2 gene; in 8 M urea-polyacrylamide gels, it yields an RNA fragment of 117 nt protected by IL-2 mRNA. The IFN- $\gamma$  probe (274 nt), transcribed from the T3 promoter, is complementary to the third exon and a portion of the third intron of the IFN- $\gamma$  gene and yields an RNA fragment of 183 nt protected by IFN- $\gamma$  mRNA. The TNF- $\beta$  probe (700 nt), transcribed from the T3 promoter, is complementary to part of exon 1, exon 2, exon 3, and portions of intron 3 and exon 4; TNF- $\beta$  mRNA protects 2 fragments of 274 and 263 nt. Sense

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RNA transcripts yielded no detectable signal upon hybridization. Antisense RNA probes for 18S rRNA (protecting 90 nt) or  $\beta$ -actin (protecting 415 nt) served as loading controls.

***Quantitative dot blot hybridization of IL-2 and IFN- $\gamma$  RNA***

- 5 PBMC from 1-ml cultures were collected and lysed in 7.5 M guanidinium-HCl. RNA, precipitated overnight in ethanol at -20°C, was dissolved into formaldehyde and incubated for 15 min at 60°C. Four serial 2-fold dilutions, made in 10x saline sodium citrate, were applied in duplicate to nitrocellulose sheets, using a 96-well dot blot apparatus. After baking in a vacuum oven at 80°C, sheets were hybridized  
10 separately with <sup>32</sup>P-labeled antisense RNA probes for human IL-2 and IFN- $\gamma$ , respectively. Exposed autoradiograms were scanned at 630 nm in an ELISA reader. RNA levels are expressed in units of A<sub>630</sub>. Serial twofold dilutions of a given RNA sample yield a linear optical density response over a 200-fold range of intensities of gene expression that is proportional to the concentration of specific RNA present in  
15 each sample [Arad *et al.* (1995) *ibid.*; Gerez *et al.*, Clin Immunol Immunopathol 58:251 (1991); Kaempfer *et al.*, J Clin Oncol 14:1778 (1996)].

***Synthesis of SEB-related peptides***

- Peptides were synthesized in the Peptide Synthesis Unit of The Hebrew University-Hadassah Medical School, Jerusalem. tBOC chemistry (tertiary butoxycarbonyl protection of amino termini) was used to prepare one batch of peptides, used for  
20 Figs. 3-5, Table 2, and trials 2 and 3 in Table 3; peptides were cleaved, and the side chain deprotected, with fluoric acid and then solubilized with 10% dimethylsulfoxide. The same amount of dimethylsulfoxide was added to control cell cultures. Fluoronylmethoxycarbonyl (Fmoc) chemistry was used to prepare a second batch  
25 of peptides, used for Figs. 6 and 7 and trial 1 in Table 3; peptides were cleaved, and the side chain deprotected, with trifluoroacetic acid. Trifluoroacetic acid-peptide salts were soluble in culture medium. In the experiment of Fig. 6, corresponding peptides from both batches were also compared and found to have equal antagonist activity. N-terminal laurylcysteine (LC-) and C-terminal cysteine (-C) were added  
30 under the same conditions used for the other amino acids. Peptides were >95% purity by HPLC.

***Proteosome or KLH coupling of peptides***

Outer membrane proteosome preparations were stored at -70°C after purification, as described [Mallett *et al.*, Infect Immun 63:2382 (1995)], from group B type 2

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*Neisseria meningitidis* by extraction of a paste of phenol-killed bacteria with a solution of 6% Empigen BB (Albright and Wilson, Whitehaven, Cumbria, UK) in 1 M calcium chloride, followed by precipitation with ethanol, solubilization in Tris-buffered saline with EDTA and 1% Empigen BB, reprecipitation with ammonium sulfate, and then resolubilization in the Tris buffer with 1% Empigen BB [Lowell *et al.* (1996) *ibid.*]. LC-peptides were coupled to proteosomes as described by Lowell *et al.* [Lowell *et al.* (1996) *ibid.*]. Maleimide-activated keyhole limpet hemocyanin (KLH) was coupled to C-terminal C residues following instructions of the supplier (Pierce).

#### 10 ***Immunization***

Rabbits were immunized intramuscularly with 100 µg of proteosome- or KLH-coupled peptides, at 0 and 3 weeks. Three bleeds were performed; data for the third bleed are shown in Table 2. BALB/c mice were immunized with 50-100 µg of proteosome- or KLH-coupled peptides, via the parenteral (intra-muscular) or intranasal routes as described by Lowell *et al.* [Lowell *et al.* (1996) *ibid.*]. Blood was collected periodically from rabbits from the ear vein, and from mice from the retroorbital plexus at 3 weeks before challenge with SEB. SEB-specific IgG was assayed by ELISA as [Lowell *et al.* (1996) *ibid.*].

#### ***SEB challenge assays of vaccine efficacy in vivo***

20 Lot 14-30 SEB was used for challenge. For parenteral (IM) or intranasal (IN) challenge, *D*-galactosamine sensitization was used in conjunction with SEB, as described [Lowell *et al.* (1996)].

#### **Example 1**

25 Peripheral blood mononuclear cells (PBMC) are used to provide a quantitative measure of the primary response of human T cells to an exotoxin, through expression of the Th1 type cytokine genes encoding IL-2, IFN-γ, and TNF-β. Gene expression is analyzed within hours after stimulation, providing a more direct and immediate measure of the action of SEB than cell proliferation which results from a complex series of events.

#### 30 ***Induction of IL-2, IFN-γ and TNF-β gene expression by SEB***

Exposure of PBMC to SEB leads to induction of IL-2 and IFN-γ mRNA, shown by quantitative dot blot hybridization (Fig. 1A-1C) and RNase protection analysis with a genomic antisense RNA probe (Fig. 1D). Both methods yield similar patterns of

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induction, characterized by a transient wave of IL-2 mRNA and more prolonged expression of IFN- $\gamma$  mRNA. TNF- $\beta$  mRNA was induced more gradually (Fig. 1E). Patterns of mRNA have thus been documented to reflect the expression of the active proteins.

## 5 Example 2

### *Design of SEB-Related Peptides*

Fourteen peptides representing particular SEB domains were synthesized (Table 1; peptides were >95% purity by HPLC) and assayed for the ability to block SEB-mediated induction of IL-2, IFN- $\gamma$  or TNF- $\beta$  gene expression. Identification of such  
10 a peptide could be useful for developing a peptide vaccine against SEB and more directly, for preventing the harmful effects of SEB on the immune response. Multiple, widely separated regions within SEB interact with the TCR on one hand and with the MHC class II molecule on the other [Swaminathan *et al.* (1992) *ibid.*; Jardetzky *et al.*, Nature **368**:711 (1994)]. Domains chosen consist of amino acid  
15 residues 13-33, 41-61, 81-93 and 208-218, essential for binding to TCR and MHC class II; residues 21-29 and 48-61, essential for binding to the TCR; and residues 13-17 and 44-52, essential for binding to MHC class II [Swaminathan *et al.* (1992) *ibid.*]. A 12-amino acid SEB domain, made up of residues 150-161, is thought not to be involved in binding to TCR or MHC class II but forms a central turn starting  
20 within  $\beta$ -strand 7 and connecting it, via short  $\beta$ -strand 8, to  $\alpha$ -helix 4 (Table 1 and Fig. 2). These sequences are found in the SEB molecule, except for two variants of that domain devised by the inventors: dodecamer p12(150-161) and decamer p10(152-161) (150-161\* and 152-161\* in Table 1, respectively).

To allow study of their immunogenicity and ability to elicit protective immunity  
25 against SEB, many of the peptides were synthesized also with an extra lauryl-cysteine residue at their N-terminus (LC-) or with an extra cysteine residue at their C-terminus (-C), as indicated in Table 1.

## Example 3

### *Lack of SEB agonist activity of SEB-derived peptides*

30 SEB agonist activity of peptides was examined by the ability to induce expression of IL-2 and IFN- $\gamma$  genes. Even when present in 200-fold higher molar amounts than SEB, no peptide exhibited significant SEB agonist activity, defined as  $\geq 2$ -fold increase in RNA over basal level (Fig. 3). When induction of mRNA for IL-2 (Fig.

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5) or IFN- $\gamma$  (not shown) was analyzed by RNase protection, pSEB(41-61), p12(150-161) and pSEB(150-161) again failed to show SEB agonist activity.

#### Example 4

##### *Peptide p12(150-161) is an SEB antagonist*

5 Antagonist activity of SEB-related peptides was defined by the ability to block SEB-mediated induction of IL-2, IFN- $\gamma$ , and/or TNF- $\beta$  gene expression in PBMC. A short, unstructured peptide would be expected to compete poorly with intact SEB whose binding is stabilized by multiple interactions with the TCR and MHC class II molecule [Swaminathan *et al.* (1992) *ibid.*; Jardetzky *et al.* (1994) *ibid.*]. However,  
10 an appropriate SEB-related peptide might compete with SEB for one of its cognate sites, preventing thereby a cooperative interaction with multiple sites.

Ability to antagonize induction of IL-2 or IFN- $\gamma$  gene expression was assayed by exposing PBMC populations to SEB in the presence of a 100- to 200-fold molar excess of an individual peptide. The resulting hybridization patterns for IL-2 and  
15 IFN- $\gamma$  RNA are shown and quantitated in Fig. 4A. Antagonist activity is seen more clearly in Fig. 4B where extent of inhibition is plotted. Most peptides failed to inhibit SEB-mediated IL-2 mRNA induction perceptibly but pronounced antagonist activity was exhibited by peptides pSEB(150-161), pSEB(152-161), p12(150-161) and p10(152-161). Dodecapeptide p12(150-161) (SEQ ID NO:2) stands out as  
20 antagonist, inhibiting expression of IL-2 mRNA by 18-fold and that of IFN- $\gamma$  mRNA by 10-fold. Peptide p10(152-161) (SEQ ID NO:4), which lacks the 2 N-terminal amino acids of p12(150-161), showed lower, yet still significant, antagonist activity. In >5 experiments, each performed with a distinct PBMC population, SEB antagonist activity of p12(150-161) ranged from 9- to 40-fold inhibition of IL-2  
25 gene induction. Corresponding extent of inhibition by p10(152-161) was up to 8-fold, other peptides remaining well below this value.

PBMC cultured with either pSEB(150-161) or p12(150-161) showed undiminished viability, as judged by trypan blue exclusion analysis and recovery of total cellular RNA. The SEB antagonist activity of these peptides thus does not result from a  
30 cytotoxic effect. Both peptides reproducibly failed to inhibit PHA-mediated induction of IL-2 and IFN- $\gamma$  genes (not shown).

The natural homolog of p12(150-161), pSEB(150-161), was less active than the variant peptide as SEB antagonist (Fig. 4). Clear differences in antagonist activity between p12(150-161) and pSEB(150-161) are seen in Figs. 5 and 6. Whereas

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- pSEB(41-61), chosen as control, failed to block induction of IL-2 and IFN- $\gamma$  mRNA by SEB, p12(150-161) yielded an almost complete inhibition (Fig. 5). pSEB(150-161) inhibited expression of IL-2 mRNA effectively at 6 hrs, but only partially at 8 hrs and reduced expression of IFN- $\gamma$  mRNA by 2 hrs but not thereafter; a stimulatory effect seen at later times (Fig. 5) was not observed consistently (cf. Fig. 6). In the experiment of Figure 6, p12(150-161) blocked induction of IL-2, IFN- $\gamma$  and TNF- $\beta$  mRNA completely, whereas pSEB(150-161) caused only partial inhibition. p12(1-161) was consistently more effective than pSEB(150-161) as SEB antagonist.
- None of the peptides homologous to toxin domains involved in the interaction with T cell receptor and/or MHC class II molecule was able to inhibit the SEB-mediated induction of human IL-2, IFN- $\gamma$ , and TNF- $\beta$  genes. By contrast, the inventors have identified 12-mer p12(150-161), resembling a region well removed from these active sites which has the capacity to completely block expression of these cytokine genes upon their induction by SEB. The sequence of this potent antagonist peptide is man-made, deviating at various positions from the corresponding sequence in SEB; indeed, when a peptide with the natural SEB sequence was used, pSEB(150-161), it was less effective as antagonist. Antagonist activity decreased upon removal of 2 N-terminal amino acids. Despite its high degree of conservation, the charge of the corresponding sequence in SEA is neutral whilst that of pSEB(150-161) or of p12(150-161), is positive. Indeed, although SEB is 68% homologous with SEC, it shows only 27% homology with SEA [Betley and Mekalanos, J Bacteriol 170:34 (1995)].
- The region covering amino acids 150-161 overlaps partially with a larger, 31-amino acid peptide, pSEB(130-160). When conjugated to KLH, pSEB(130-160) inhibited the SEB-induced proliferation of mixed cultures of human peripheral blood monocytes and lymphocytes by 2- to 4-fold but was not unique in this property, since peptides overlapping with other SEB domains, covering amino acids 1-30, 61-92, 93-112, 151-180, 171-200 and 191-220, had a similar inhibitory effect [Jett *et al.* (1994) *ibid.*]. Although pSEB(130-160) was able to inhibit binding of SEB to human lymphocytes [Jett *et al.* (1994) *ibid.*, Komisar *et al.*, Infect Immun 62:4775 (1994)] it was shown that a smaller overlapping peptide, pSEB(150-162), failed to inhibit binding of SEB to HUT-78 cells, a human T cell line, as studied by fluorescence, whereas another peptide, pSEB(90-114), inhibited by 2-fold. Wang *et al.* [(1993) *ibid.*] showed that a synthetic peptide encoding the carboxy-terminal 41 amino acids of the superantigenic pep M5 protein of *S. pyogenes* inhibited pep M5-

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mediated T cell proliferation. They commented with respect to pSEB(152-160) that "most studies seem to indicate that this region does not contribute to mitogenicity" while emphasizing in this context that "immunologic function is not determined solely by the primary amino acid structure of a particular region but is influenced by the context in which it is located" and indicating that the longer amino acid sequence may contribute to  $\alpha$ -helix amphi-philicity. The lack of inductive activity of pSEB(150-161) or p12(150-161) is shown in Fig. 3 for the IL-2 and IFN- $\gamma$  genes and again for the IL-2 gene in Fig. 5. Indeed, the N-terminal 138 amino acids of SEB, which exclude the domain of pSEB(150-161), are sufficient for mitogenic activity [Buelow *et al.*, J Immunol **148**:1 (1992); Kappler *et al.*, J Exp Med **175**:387 (1992)]. Thus, it would be expected from these earlier studies that short peptides, especially in the region of pSEB(150-161), will not inhibit the action of SEB. Contrary to this expectation, the results of Figs. 4-6 show that pSEB(150-161) and the non-natural p12(150-161) are powerful SEB antagonists.

## 15 Example 5

### *Enhancement of SEB antagonist activity*

With PBMC populations from occasional healthy donors, it was observed that p12(150-161) was only weakly or not inhibitory to SEB. Such an experiment is illustrated in Fig. 7. Whereas p12(150-161) did not inhibit induction of IL-2 and IFN- $\gamma$  mRNA, both its dimer and trimer forms were strongly inhibitory (shown for IFN- $\gamma$  in Fig. 7A). Fig. 7B shows that even when diluted 100-fold, the dimer still gave a detectable inhibition.

A similar rise in efficacy was observed when p12(150-161) was cyclized with terminal cysteines (Cys-p12(150-161)) (Fig. 8). Given the oxidizing conditions in aqueous solution, this peptide will tend to cyclize by forming an intramolecular disulfide bridge in a zero-order reaction; multimeric forms generated by disulfide bridges between separate peptide molecules will tend to be rarer as they result from a higher order reaction. Whereas p12(150-161) was weakly or not inhibitory at 8 hrs (Figs. 8A and 8C), Cys-p12(150-161) showed significant SEB antagonist activity, even upon tenfold dilution (Figs. 8B and 8D). An enhancement in SEB antagonist activity was also obtained by addition of a D-Ala residue at both N- and C-termini (see Figs. 11 and 12 below).

## Example 6



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***Broad-spectrum pyrogenic exotoxin antagonist activity***

The SEB 150-161 domain is conserved among pyrogenic toxins. The sequence of p12(150-161), shown in SEQ ID NO: 2, differs in several positions from the corresponding sequence in SEB, TNKKKVTAQELD found in pSEB(150-161), but shared KKK and QELD motifs are spaced equally in both peptides. Residues T150, K152, E159 and D161 of this SEB domain are conserved among all staphylococcal enterotoxins [Swaminathan *et al.* (1992) *ibid.*]. Indeed, domain 150-161 of SEB is highly conserved among pyrogenic toxins, with 10/12 identities for SEA, SEC1, SEC2, and SPE A and 9/12 for SEE [Bohach and Schlievert (1987) *ibid.*; Couch *et al.* (1988) *ibid.*; Bohach and Schlievert (1989) *ibid.*]. All of these toxins contain the residues underlined above [Swaminathan *et al.* (1992) *ibid.*; Bohach and Schlievert (1987) *ibid.*; Couch *et al.* (1988) *ibid.*; Bohach and Schlievert (1989) *ibid.*].

The prediction that SEB antagonist peptides may have wider antagonist activity was tested. Figure 9 shows that pSEB(150-161) failed to inhibit the induction of IL-2 mRNA by TSST-1, although it strongly reduced the induction of this mRNA by SEB. As seen in Fig. 10, moreover, p12(150-161) effectively inhibited the induction by SEB of waves of IL-2 mRNA (A) and IFN- $\gamma$  mRNA (B) but had no significant effect on their induction by SEA.

The results of Figs. 9 and 10 lend support to the earlier conclusion that the antagonist activity of pSEB(150-161) and p12(150-161) for SEB does not result from a cytotoxic effect on the cells examined because these peptides fail to inhibit induction by TSST-1 and SEA.

These results would lead a man of the art to the conclusion that the SEB antagonist activity of the peptides examined does not extend to two less related toxins, TSST-1 and SEA.

Nevertheless, in contrast to the results of Figs. 9 and 10 which suggest narrow specificity for the antagonist peptides, Figs. 11 and 12 show that broad-spectrum antagonist activity is exhibited by derivatives of p12(150-161), the dimer described in Fig. 7 and p12(150-161) carrying D-Ala at both N- and C-termini.

Figure 11 shows induction of IL-2 and IFN- $\gamma$  mRNA in a PBMC population where peptide p12(150-161) did not significantly inhibit SEB-induced gene expression. However, both dimer and p12(150-161) carrying D-Ala at both N- and C-termini were effective as antagonist, with the latter showing highest antagonist activity. Despite the low homology between corresponding regions in SEB and TSST-1 (Fig.

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12), the *D*-Ala form also inhibited induction of IL-2 mRNA by TSST-1 (6-hour point in Fig. 11).

Figure 12 shows, for another PBMC population, induction of IL-2 mRNA by SEB, by TSST-1 as well as by SEA. Induction of IL-2 mRNA by all three toxins was inhibited by p12(150-161) carrying *D*-Ala at both *N*- and *C*-termini. For SEB and TSST-1, data with the dimer of p12(150-161) are also shown. Induction of IL-2 mRNA by both toxins was inhibited by the dimer.

These results show that the SEB antagonist activity of the p12(150-161) dimer and *D*-Ala forms extends to other members of the pyrogenic exotoxin family, TSST-1 and SEA, indicating their potential broad-spectrum toxin antagonist activity. The dimer, Cys and *D*-Ala forms are each more powerful as antagonist than p12(150-161) (Figs. 7, 8 and 10). Independent of the basis for enhanced antagonist activity in p12(150-161) derivatives (they may be more stable, exhibit higher affinity for a target, or both), these results show that the sequence of p12(150-161) has the potential of being a broad-spectrum pyrogenic exotoxin antagonist.

#### Example 7

Dodecamer antagonist peptide p12(150-161) elicits, in rabbits, antibodies that protect human T cells, capable of expressing IL-2 and IFN- $\gamma$  genes, from activation not only by SEB but also by SEA and TSST-1. This finding indicates that used as vaccine, the peptide has the potential to confer broad-spectrum protective immunity.

#### *Antiserum against p12(150-161) blocks the action of SEB, SEA and TSST-1*

Antibodies raised against an SEB-related peptide might bind to SEB and modulate its action. To allow study of their immunogenicity and ability to elicit protective immunity against SEB, most peptides were synthesized also with an added *N*-terminal lauryl-Cys or *C*-terminal Cys (Table 1), to permit their linkage to proteosomes or KLH adjuvant, respectively [as described by Lowell *et al.* (1996) *ibid.*], in order to facilitate generation of antibodies. In addition, alum may be used as an immunization adjuvant directly with non-linked peptide, or after linking a peptide to proteosomes or to KLH [Lowell *et al.* (1996) *ibid.*].

Sera from rabbits immunized with individual peptides were titrated for their ability to bind SEB. Most of the peptides in Table 1 proved immunogenic by this parameter but greatly differed in titer of serum anti-SEB IgG achieved. Thus, serum against pSEB(13-33)C had a titer of 102,400 while corresponding titers for pSEB(81-93),

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pSEBLC(41-61) and p12LC(150-161) were 50, 1,600 and 1,600, respectively (Table 2). On the basis of the results in Table 2, one would not select p12(150-161) for use as a peptide vaccine but instead, prefer to use pSEB(13-33)C.

None of the antisera raised against individual SEB-related peptides had any SEB agonist activity, defined by ability to induce IL-2 or IFN- $\gamma$  mRNA (not shown). Ability to inhibit SEB-mediated induction of these genes is examined in Fig. 13. Normal rabbit serum did not affect this induction (not shown). Consistent with its high titer of anti-SEB IgG, anti-pSEB(13-33)C serum partially inhibited induction of IL-2 and IFN- $\gamma$  mRNA. However, despite its far lower titer, anti-p12LC(150-161) serum completely blocked the induction of both genes (Figs. 13A and 13B).

Antisera against pSEB(81-93) and pSEBLC(41-61), which had low titers of anti-SEB IgG, not only failed to inhibit expression of IL-2 and IFN- $\gamma$  mRNA but significantly stimulated their SEB-mediated induction (Figs. 13A and 13B). This result was unexpected. This finding raises the possibility that elicitation of SEB-sensitizing antibodies, for example, by a toxoid [Lowell *et al.*, Infect Immun 64:1706 (1996a); Lowell *et al.*, Infect Immun 64: 4686 (1996b)] or mutant toxin vaccine [Stiles *et al.*, Infect. Immun. 63:1229 (1995); Woody *et al.*, Vaccine 15:133 (1997)], could lead, in a polyclonal antibody reaction, to exacerbation of toxic immune responses in SEB-exposed individuals. Antibodies raised against peptide p12(150-161), on the other hand, are free of exacerbating properties as measured *in vitro*, indicating that the peptide, or its derivatives, may serve as safer anti-toxin vaccine.

There was a striking lack of correlation between the ability of anti-peptide sera to bind SEB and to block SEB action. Antisera against pSEBLC(41-61) and p12LC(150-161) showed identical anti-SEB IgG titers yet affected induction of IL-2 and IFN- $\gamma$  genes by SEB in an opposite manner, stimulation vs. complete inhibition. Apparently, certain antibodies effectively potentiate SEB action while others block it. As judged by serial dilution, inhibition of SEB-mediated gene induction was also up to 30-fold more sensitive for detecting blocking antibodies than binding of IgG to SEB.

In an earlier study, rabbit sera raised against SEB peptides 113-144, 130-160, 151-180 and 171-200 each reduced SEB-induced lymphocyte proliferation weakly ( $\leq 2.5$ -fold), apparently in a nonspecific manner [Jett *et al.* (1994) *ibid.*].

Antibodies against p12(150-161) have broad-spectrum toxin blocking activity. In Figs. 14A and 14B, SEA was used as inducer. Again, sera raised against

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p12LC(150-161) and pSEB(13-33)C strongly inhibited IL-2 and IFN- $\gamma$  gene expression. As for SEB (Fig. 13A), sera raised against pSEB(81-93) or pSEBLC(41-61) failed to inhibit but instead, stimulated induction of IL-2 mRNA by up to 7-fold over the SEA control. Antiserum against pSEB(81-93) also stimulated expression of IFN- $\gamma$  mRNA.

Antibodies raised against p12(150-161) and pSEB(21-33)C likewise inhibited the action of the even less related toxin, TSST-1 (Fig. 15).

Hence, as judged from inhibition of IL-2 and/or IFN- $\gamma$  gene expression analysis, protective activity of antibodies elicited by p12(150-161) is not narrowly restricted in range of pyrogenic exotoxins (Figs. 13-15). These results suggest that p12(150-161) is potentially a broad-spectrum peptide vaccine against pyrogenic exotoxins.

#### Example 8

##### *Protective effect of SEB-related peptides as SEB vaccine in mice*

The finding that antiserum against p12(150-161) blocks the action of SEB in human PBMC suggested its potential as peptide vaccine. To examine this point, the D-galactosamine-treated mouse model was used [Lowell *et al.* (1996a) *ibid.*]. Mice were repeatedly immunized with individual peptides and then challenged with a lethal dose of SEB. Table 3 details the results of 3 separate trials.

In the first, formalin-inactivated intact SEB toxoid protected 20% of the mice against a lethal dose of SEB, either upon parenteral (i.m.) or intranasal vaccination. The fact that SEB toxoid did not afford complete protection as reported [Lowell *et al.* (1996a) *ibid.*] shows that the conditions of toxin challenge were more severe in the present experiment. In this trial, proteosome-coupled p12(150-161) yielded 10% protection. Relative to SEB toxoid, p12(150-161) thus did show protective activity.

In the second trial, the ability of proteosome-coupled pSEB(150-161) and p12(150-161) to provide immune protection against SEB challenge was compared to that of several larger, synthetic peptides [Jett *et al.* (1994)] derived from the SEB amino acid sequence, including pSEB(130-160) which overlaps almost completely with pSEB(150-161) and p12(150-161). None of the larger peptides showed detectable protective activity against intranasal challenge with SEB, yet both pSEB(150-161) and p12(150-161) afforded protection, evident from 22-29% survival (Table 3). pSEB(150-161) also elicited protective immunity (22%) when vaccination was by the intramuscular route (trial 3) rather than intranasally (trial 2). By contrast, two larger peptides, pSEB(130-160) and pSEB(151-180) that overlap in part with

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pSEB(150-161), failed to give protection. Peptide pSEB(13-33)C, moreover, though able to elicit in rabbits a high titer of IgG that inhibit the action of SEB, SEA and TSST-1 on human T cells, capable of expressing IL-2 and IFN- $\gamma$  genes, *in vitro* (Table 2 and Figs. 13-15), was not protective in mice.

- 5 Despite the severity of challenge with SEB toxin in these early trials, two SEB-related peptides exhibiting SEB antagonist activity, pSEB(150-161) and p12(150-161), also showed activity as SEB vaccine. Protective effect of SEB-related peptides tended to correlate with SEB antagonist activity on human PBMC *in vitro* (Figs. 4-6) rather than with the ability to elicit SEB-binding IgG (Table 2). In trials 2 and 3,
- 10 murine SEB-binding IgG titers were 50-200 for all peptides and did not correlate with protection (Table 3). Significantly, peptide pSEB(13-33)C, though able to elicit in rabbits a high titer of IgG that bind SEB and inhibit SEB action on PBMC *in vitro* (Table 2 and Fig. 13, was neither active as SEB antagonist (Fig. 4) nor protective in mice (Trial 3).
- 15 These examples show that it is possible to design an effective pyrogenic exotoxin antagonist. It is surprising that a linear, unstructured dodecapeptide, such as p12(150-161) or pSEB(150-161), can compete effectively with the intact, folded 239-amino acid SEB protein chain which interacts at multiple domains with regions in the MHC Class II molecule and in the T cell receptor. Such concerted interaction
- 20 would lead to far higher affinity of binding for intact SEB *vis-a-vis* the peptides. Yet, as shown clearly in Figs. 4-6, the action of SEB on human PBMC can be neutralized almost completely by the dodecapeptides, especially by p12(150-161). This first-generation antagonist, moreover, elicited in rabbits the production of antibodies that block the action of SEB on human T cells.
- 25 Subtle changes in peptide composition had marked effects on its SEB antagonist activity (Figs. 4-6), indicating that substantial improvement is possible by methods known to a man of the art. In principle, significantly enhanced pyrogenic exotoxin antagonist activity can be obtained by generating dimers or multimeric forms (as shown in Fig. 7) or by constraining conformation, by use of disulfide bridges (as
- 30 shown in Fig. 8), by internal bridges, short-range cyclizations or other means. In principle, improvement of the stability of the described peptides by these means will also render them more effective as peptide vaccine against pyrogenic toxins.

For example, the amino acid sequence of these dodecapeptides could also be extended stepwise at their *N*- or *C*-termini, or both, either with the natural SEB

35 amino acid sequence, or with a *D*-amino acid that will tend to render the peptide less

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amenable to digestion by L-amino-acid-specific proteases (as shown for *D*-Ala in Figs. 11 and 12) or with aromatic residues such as Trp to enhance the hydrophobicity of the resulting peptide, or with a random sequence of amino acids followed by selection using existing methods, for example, phage display, in order to obtain peptide(s) with enhanced antagonist activity and/or immunoprotective properties. Immunogenicity may be enhanced, for example, by use of *in vitro* reconstituted MHC/peptide complex as described by Sakita *et al.* [Sakita *et al.*, J. Immunol. Methods **192**:105 (1996)].

There are few examples of short peptides able to bind with sufficiently high affinity to a receptor in order to mimic the binding of the full-length ligand or to block its action. A T-cell receptor antagonist peptide of 4 amino acids was able to inhibit clinical disease progression in experimental allergic encephalomyelitis mediated by a diverse T cell repertoire [Kuchroo *et al.*, J. Immunol. **153**:3326 (1994)]. Peptides derived from the predicted helical region of MHC class II molecules may interact directly with T cell receptors: one such a peptide, a 16-mer, appeared capable of modulating immune responses in a physiologically significant manner [Williams *et al.*, Immunol Res **11**:11, (1992)]. In another example, a synthetic, 22-amino-acid segment of the human IFN- $\gamma$  receptor was found to antagonize the action of IFN- $\gamma$  [Seelig *et al.*, J Biol Chem **270**:9241 (1995)]. The linear forms of peptides generally lack a stable conformation in solution. Interaction with the cognate binding site on a receptor may induce folding of the peptide to mimic conformation in the native protein. Significant improvement in binding affinity can be achieved by generating dimers or multimeric forms of the peptide (Fig. 7) or by constraining conformation, for example, through cyclization (Fig. 8). Thus, by dimerizing bioactive peptides based on an antibody hypervariable region sequence, higher affinity binding was produced; an optimized cyclic peptide showed up to 40-fold enhanced affinity when compared to the linear form [Williams *et al.*, J Biol Chem **266**:5182, (1991)]. A hexapeptide, once cyclized by oxidizing a Cys-hexapeptide-Cys form, showed a higher avidity for the collagen receptor than the more flexible linear structure [Cardarelli *et al.*, J Biol Chem **267**:23159 (1992)]. A synthetic 15-amino-acid peptide mimic of plasma apolipoprotein E failed to bind to the low density lipoprotein receptor but its dimeric form was active in binding; a trimer, moreover, had 20-fold greater activity than the dimer [Dyer and Curtiss, J Biol Chem **266**:22803 (1991)]. In addition to affecting peptide conformation and thus enhancing its binding affinity and/or immunogenicity, multimerization or cyclization of a peptide may also enhance its biostability, thus enhancing its efficacy

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as vaccine. These examples provide to a man of the art methods to improve upon the antagonist activity of SEB-related peptides as detailed in this invention and through this criterion, potential vaccine efficacy.

5 Methods for generating multimeric or cyclic forms of peptides exist, as by direct synthesis (Fig. 7). Another approach is to generate two terminal Cys residues as described above [Cardarelli *et al.* (1992) *ibid.*]; their oxidation will yield both cyclic and multimeric forms, mostly dimers (Fig. 8). An efficient procedure for the preparation of protected cyclized and protected symmetrical dimeric peptide disulfides by oxidative detachment from a support has been described [Rietman *et al.*, Int J Pept Protein Res **44**:199 (1994)]. Fully cyclic forms of peptides will lack free termini but cyclic peptides can be made by internal bridges, or short-range cyclizations [Toniolo, Int J Peptide Protein Res **35**:287 (1990); Gilon *et al.*, Biopolymers **31**:745 (1991)] to allow synthesis of LC- or -C termini.

15 The experimental approach employed here used expression of human cytokine genes *in vitro* as a tool for designing both a broad-spectrum pyrogenic toxin antagonist and a prototypical peptide vaccine. This molecular approach is far more rapid than conventional methods that are based solely on animal tests, allowing evaluation of the biological properties of a candidate peptide well before moving on to animal studies for analysis of vaccine efficacy, prophylactic and therapeutic activity. Specifically, analysis of pyrogenic toxin antagonist activity on human T cells can be used to direct effective vaccine development, even before ability to elicit antibodies is studied in animals.

25 Efficacy of potential vaccine candidates can be evaluated not only by their pyrogenic toxin antagonist activity but also by their ability to elicit production of antibodies in rabbits (shown for SEB in Table 2) that can block the harmful action of pyrogenic exotoxins on human lymphoid cells (Figs. 13-15), independently of their ability to bind toxin. The examples show that assay of the ability of anti-peptide antibodies to block the action of SEB on human cytokine gene expression is far more sensitive than assay of the ability to bind SEB (Table 2 and Figs. 13 and 30 14. At least certain antisera showing very high anti-SEB IgG titers (Table 2) are not necessarily protective against the toxin. This result casts doubt on the relevance of IgG assays for evaluating or predicting immunoprotection against SEB, as also shown in Table 3, trials 2 and 3.

35 In order to vaccinate humans effectively against one or more pyrogenic toxins, there is a need to assess the efficacy of vaccination. This requirement is independent of

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- the nature of the vaccine, whether a toxoid, a mutant toxin or a peptide. However, unlike experimental animals (Table 3), humans cannot be challenged with toxin to test if the vaccination was effective. Therefore, it is desirable to use a surrogate marker instead, preferably one based on human T cell activation. Such a marker is
- 5 provided through the method used in Figs. 13-15, which measures the ability of serum from an immunized individual to antagonize toxin-mediated activation of human T cells. Given the fact that humans are far more sensitive than mice to staphylococcal toxins, use of human T cells as shown in Figs. 13 to 15 has advantages over use of murine cells [Stiles *et al.*, Infect Immun 63:1229 (1995)].
- 10 Toxin-mediated activation of T cells is preferably measured by the induction of IL-2, IFN- $\gamma$  or TNF- $\beta$  gene expression, which yields information on the response of these genes within a few hours after their induction, long before any effect on cell proliferation.
- SEB-related peptides with demonstrated SEB antagonist activity, p12(150-161) and
- 15 pSEB(150-161), carry sequence elements that are highly conserved amongst pyrogenic toxins. Through improved peptide design, including introduction of structural constraints, peptides may thus be generated that exhibit broader antagonist activity (Figs. 11, 12). A peptide antagonist able to protect against a range of related enterotoxins would be far more valuable than a narrowly active one.
- 20 Rabbit sera raised against p12(150-161) were equally able to block the ability of SEB, SEA or TSST-1 to induce expression of IL-2 and IFN- $\gamma$  genes (Figs. 13-15). These results show a potential for broader protective immunity. A peptide vaccine able to impart protective immunity against a range of related enterotoxins would be far more valuable than a narrowly active one.
- 25 An unexpected finding, shown in Figs. 13 and 14, is that antisera against certain SEB peptides not only fail to block the action of SEB on human lymphoid cells, but actually stimulate it significantly. This result should alert one to the possibility that vaccination with such peptide domains, including those present in SEB toxoid or other derivatives of the SEB toxin molecule, could sensitize an exposed person to
- 30 the lethal effects of SEB and other pyrogenic exotoxins, for example SEA, rather than protect him.



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**Table 1**  
**SEB-related peptides prepared for this study**

Position	Amino acid sequence	LC-	-C
13-24	KSSKFTGLMENM	+	-
13-33	KSSKFTGLMENMKVLYDDNHV	+	+
21-33	MENMKVLYDDNHV	+	+
41-53	IDQFLYFDLIYSI	-	-
41-61	IDQFLYFDLIYSIKDTKLGNY	+	+
51-61 <sup>@</sup>	YSIKDTKLGNY	-	+
53-61	IKDTKLGNY	+	-
81-92	YVDVFGANYYYQ	+	-
81-93	YVDVFGANYYYQC	-	-
150-161	TNKKKVTAQELD	+	-
150-161*	<u>Y</u> NKKK <u>A</u> T <u>V</u> QELD	+	-
152-161	KKKVTAQELD	-	-
152-161*	KKK <u>A</u> T <u>V</u> QELD	-	-
208-218	FDQSKYLMMYN	+	+

LC- Lauryl-cysteyl residue added at N-terminus

-C Cysteyl residue added at C-terminus

5 <sup>@</sup> Only the -C form was studied

\* Variant of the natural SEB sequence

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Table 2

**Anti-SEB antibody titers in sera from rabbits immunized with SEB-related peptides**

<b>SEB Peptide</b>	<b>SEB IgG Titer</b>
pSEBLC(13-24)	800
pSEBLC(13-33)	800
pSEBLC(21-33)	100
pSEBLC(41-61)	1,600
pSEBLC(53-61)	1,600
pSEBLC(81-92)	1,600
p12LC(150-161)	1,600
pSEBLC(208-218)	1,600
pSEB(13-33)C	102,400
pSEB(21-33)C	25,600
pSEB(41-61)C	200
pSEB(51-61)C	400
pSEB(81-93)	50
pSEB(208-218)C	200

See Table 1 for explanation of SEB peptides. For immunization, peptides carrying  
 5 LC at their N-terminus were coupled to proteosomes; peptides carrying C at their C-terminus were coupled to KLH. Sera were assayed for IgG able to bind SEB.

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**Table 3.**  
**Protective Effect of SEB Peptides in Mice**

<b>Trial</b>	<b>Vaccine</b>	<b>No. of Animals</b>	<b>Vaccine Route</b>	<b>Challenge Route</b>	<b>Challenge (<math>\mu</math>g SEB)</b>	<b>IgG Titer</b>	<b>Percent Survival</b>
<b>1</b>	p12LC(150-161)	10	IM	IM	25		10
	p12LC(150-161)	10	IN	IM	25		10
	SEB Toxoid	10	IM	IM	25		20
	SEB Toxoid	10	IN	IM	25		20
<b>2</b>	pSEBLC(150-161)	7	IN	IN	350	200	29
	p12LC(150-161)	9	IN	IN	350	200	22
	pSEBLC(93-112)	9	IN	IN	350	200	0
	pSEBLC(130-160)	9	IN	IN	350	200	0
	pSEBLC(191-220)	9	IN	IN	350	200	0
	pSEB(191-220)C	9	IN	IN	350	50	0
	Control	5	IN	IN	350	100	0
<b>3</b>	pSEBLC(150-161)	9	IM	IN	350	200	22
	pSEB(13-33)C	9	IM	IN	350	100	0
	pSEBLC(93-112)	9	IM	IN	350	200	0
	pSEBLC(130-160)	9	IM	IN	350	100	0
	pSEBLC(151-180)	9	IM	IN	350	200	0
	pSEBLC(191-220)	9	IM	IN	350	200	0
	pSEB(191-220)C	9	IM	IN	350	50	0

Groups of mice were vaccinated with SEB-related peptides or SEB toxoid as indicated. SEB toxoid [Lowell *et al.*, (1996) *ibid.*], peptides pSEB(93-112), pSEB(130-160), pSEB(151-180), pSEB(191-220) and pSEB(191-220)C [Jett *et al.*, (1994) *ibid.*] and preparation of LC-derivatives for coupling to proteosomes [Lowell *et al.*, (1996) *ibid.*], were described elsewhere. In trial 1, 2 vaccinations were given 2 weeks apart, with SEB challenge after 1 month. In trials 2 and 3, 3 vaccinations were given at 2-week intervals, followed by a boost after 6 weeks and SEB challenge 2 weeks later. IgG were determined 3 weeks before challenge. IM, intramuscular; IN, intranasal.

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Table 4 lists for each of the SEQ ID NOs' of the sequence listing, the corresponding alternative notation used in the specification.

**Table 4**  
**Identification of SEB related peptides by their SEQ. ID. No.**

SEQ ID NO:	Alternative notation	Sequence
SEQ ID No. 1	pSEB(150-161)	TNKKK VTAQELD
SEQ ID No. 2	p12(150-161)	YNKKKATVQELD
SEQ ID No. 3	pSEB(152-161)	KKKATVQELD
SEQ ID No. 4	p10(152-161)	KKK VTAQELD
SEQ ID No. 5	pSEBLC(150-161)	lcTNKKK VTAQELD
SEQ ID No. 6	p12LC(150-161)	lcYNKKKATVQELD
SEQ ID No. 7	Dimer	YNKKKATVQELDYNKKKATVQEL D
SEQ ID No. 8	Trimer	YNKKKATVQELDYNKKKATVQEL DYNKKKATVQELD
SEQ ID No. 9	Cys-p12(150-161)	CYNKKKATVQELDC
SEQ ID No. 10	D-Ala	daYNKKKATVQELDda
SEQ ID No. 11	Ac-p12(150-161)	acYNKKKATVQELDda
SEQ ID No. 12	SEB	ESQPDPKPDELHKSSKFTGLMENM KVLYDDNHVSAINVKSIDQFLYFD LIYSIKDTKLGNYDNVRVEFKNKD LADKYKDKYVDVFGANYYYQCYF SKKTNDINSHETDKRKTCTMYGGVT EHNGNQLDKYRSITVRVFEDGKNL LSFDVQTNKKK VTAQELDYLTRH YLVKNKKLYEFNNSPYETGYIKFI ENENSWYDMMPAPGDKFDQSKY LMMYNDNKMVDSKDVKIEVYLTT KKK

5 lc, *N*-lauryl-cysteyl; da, *D*-Ala; ac, *N*-acetyl.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

- (A) NAME: YISSUM RES. DEVELOP. CO. OF THE HEBREW UNIV.  
OF JERUSALEM  
(B) STREET: 46 Jabotinsky street  
(C) CITY: Jerusalem  
(E) COUNTRY: ISRAEL  
(F) POSTAL CODE (ZIP): 91042  
(G) TELEPHONE: 972-2-5634880  
(H) TELEFAX: 972-2-5660331

(ii) TITLE OF INVENTION: BROAD SPECTRUM PYROGENIC EXOTOXINS  
ANTAGONISTS AND VACCINES

## (iii) NUMBER OF SEQUENCES: 12

## (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Thr Asn Lys Lys Lys Val Thr Ala Gln Glu Leu Asp  
1                      5                      10

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Tyr Asn Lys Lys Lys Ala Thr Val Gln Glu Leu Asp  
1                      5                      10

## (2) INFORMATION FOR SEQ ID NO: 3:

## (i) SEQUENCE CHARACTERISTICS:

43

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Lys Lys Lys Ala Thr Val Gln Glu Leu Asp  
1                      5                      10

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 10 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Lys Lys Lys Val Thr Ala Gln Glu Leu Asp  
1                      5                      10

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 13 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /label= X  
/note= "N-lauryl cysteine residue"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Xaa Thr Asn Lys Lys Lys Val Thr Ala Gln Glu Leu Asp  
1                      5                      10

(2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 13 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /label= X  
/note= "N-lauryl cysteine"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Xaa Tyr Asn Lys Lys Lys Ala Thr Val Gln Glu Leu Asp  
1 5 10

## (2) INFORMATION FOR SEQ ID NO: 7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Tyr Asn Lys Lys Lys Ala Thr Val Gln Glu Leu Asp Tyr Asn Lys Lys  
1 5 10 15

Lys Ala Thr Val Gln Glu Leu Asp  
20

## (2) INFORMATION FOR SEQ ID NO: 8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Tyr Asn Lys Lys Lys Ala Thr Val Gln Glu Leu Asp Tyr Asn Lys Lys  
1 5 10 15

Lys Ala Thr Val Gln Glu Leu Asp Tyr Asn Lys Lys Lys Ala Thr Val  
20 25 30

Glu Leu Asp  
35

## (2) INFORMATION FOR SEQ ID NO: 9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

- (A) NAME/KEY: Disulfide-bond
- (B) LOCATION: 1..14
- (D) OTHER INFORMATION: /label= X  
/note= "Disulfide bond formed by the two external  
Cys residues."

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 14
- (D) OTHER INFORMATION: /label= X  
/note= "Extension with a cysteine residue."

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /label= X  
/note= "Extension with a cysteine residue"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Xaa Tyr Asn Lys Lys Lys Ala Thr Val Gln Glu Leu Asp Xaa  
1 5 10

## (2) INFORMATION FOR SEQ ID NO: 10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /label= X  
/note= "D-alanine residue."

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 14
- (D) OTHER INFORMATION: /label= X  
/note= "D-alanine residue"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Xaa Tyr Asn Lys Lys Lys Ala Thr Val Gln Glu Leu Asp Xaa  
1 5 10

## (2) INFORMATION FOR SEQ ID NO: 11:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown



(ii) MOLECULE TYPE: peptide

**(ix) FEATURE:**

(A) NAME/KEY: Modified-site

(B) LOCATION: 1

(D) OTHER INFORMATION: /label= X

```
/note= "N-acetyl group"
```

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 14

(D) OTHER INFORMATION: /label= X

```
/note= "D-alanine residue"
```

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Xaa Tyr Asn Lys Lys Lys Ala Thr Val Gln Glu Leu Asp Xaa  
1                      5                      10

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 239 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Staphylococcus aureus*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Glu Ser Gln Pro Asp Pro Lys Pro Asp Glu Leu His Lys Ser Ser Lys  
1                5                10                15

Phe Thr Gly Leu Met Glu Asn Met Lys Val Leu Tyr Asp Asp Asn His  
20 25 30

Val Ser Ala Ile Asn Val Lys Ser Ile Asp Gln Phe Leu Tyr Phe Asp  
35 40 45

Leu Ile Tyr Ser Ile Lys Asp Thr Lys Leu Gly Asn Tyr Asp Asn Val  
50 55 60

Arg Val Glu Phe Lys Asn Lys Asp Leu Ala Asp Lys Tyr Lys Asp Lys  
65                      70                      75                      80

Tyr Val Asp Val Phe Gly Ala Asn Tyr Tyr Tyr Gln Cys Tyr Phe Ser  
85 90 95

Lys Lys Thr Asn Asp Ile Asn Ser His Glu Thr Asp Lys Arg Lys Thr  
100 105 110

Cys Met Tyr Gly Gly Val Thr Glu His Asn Gly Asn Gln Leu Asp Lys  
115 120 125

Tyr Arg Ser Ile Thr Val Arg Val Phe Glu Asp Gly Lys Asn Leu Leu

130	135	140
Ser Phe Asp Val Gln Thr Asn Lys Lys Lys Val Thr Ala Gln Glu Leu		
145	150	155 160
Asp Tyr Leu Thr Arg His Tyr Leu Val Lys Asn Lys Lys Leu Tyr Glu		
	165	170 175
Phe Asn Asn Ser Pro Tyr Glu Thr Gly Tyr Ile Lys Phe Ile Glu Asn		
	180	185 190
Glu Asn Ser Phe Trp Tyr Asp Met Met Pro Ala Pro Gly Asp Lys Phe		
	195	200 205
Asp Gln Ser Lys Tyr Leu Met Met Tyr Asn Asp Asn Lys Met Val Asp		
	210	215 220
Ser Lys Asp Val Lys Ile Glu Val Tyr Leu Thr Thr Lys Lys Lys		
225	230	235

**CLAIMS:**

1. A peptide comprising an amino acid sequence substantially homologous to the amino acid sequence of a fragment of a pyrogenic exotoxin, and derivatives of said peptide capable of eliciting protective immunity against toxic shock induced by a pyrogenic exotoxin or by a mixture of pyrogenic exotoxins.
2. A peptide comprising an amino acid sequence substantially homologous to the amino acid sequence of a fragment of a pyrogenic exotoxin, and derivatives of said peptide capable of antagonizing toxin-mediated activation of T cells.
3. A peptide according to claim 1 or 2 wherein said pyrogenic exotoxin is a bacterial exotoxin.
4. A peptide according to claim 3 wherein said exotoxin is produced by *Staphylococcus aureus* or *Streptococcus pyogenes*.
5. A peptide according to claim 4 comprising an amino acid sequence substantially homologous to the amino acid sequence of a fragment of *Staphylococcal aureus* enterotoxin B (SEB).
6. A peptide according to claim 5 comprising the amino acid sequence shown in SEQ ID NO:1 and derivatives thereof, capable of eliciting protective immunity against toxic shock induced by at least one pyrogenic exotoxin and/or of antagonizing toxin-mediated activation of T cells.
7. A peptide according to claim 6 having the amino acid sequence shown in SEQ ID NO:1 and derivatives thereof, capable of eliciting protective immunity against toxic shock induced by at least one pyrogenic exotoxin and/or of antagonizing toxin-mediated activation of T cells.
8. A peptide according to claim 5 comprising the amino acid sequence shown in SEQ ID NO:2 and derivatives thereof, capable of eliciting protective immunity against toxic shock induced by at least one pyrogenic exotoxin and/or of antagonizing toxin-mediated activation of T cells.
9. A peptide according to claim 8 having the amino acid sequence shown in SED ID NO:2 and derivatives thereof, capable of eliciting protective immunity against toxic shock induced by at least one pyrogenic exotoxin and/or of antagonizing toxin-mediated activation of T cells.

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10. A peptide according to claim 5 comprising the amino acid sequence shown in SEQ ID NO:3 and derivatives thereof, capable of eliciting protective immunity against toxic shock induced by at least one pyrogenic exotoxin and/or of antagonizing toxin-mediated activation of T cells.
11. A peptide according to claim 10 having the amino acid sequence shown in SEQ ID NO:3 and derivatives thereof, capable of eliciting protective immunity against toxic shock induced by at least one pyrogenic exotoxin and/or of antagonizing toxin-mediated activation of T cells.
12. A peptide according to claim 5 comprising the amino acid sequence shown in SEQ ID NO:4 and derivatives thereof, capable of eliciting protective immunity against toxic shock induced by at least one pyrogenic exotoxin and/or of antagonizing toxin-mediated activation of T cells.
13. A peptide according to claim 12 having the amino acid sequence shown in SEQ ID NO:4 and derivatives thereof, capable of eliciting protective immunity against toxic shock induced by at least one pyrogenic exotoxin and/or of antagonizing toxin-mediated activation of T cells.
14. A peptide according to any one of claims 1, 3 to 6, 8, 10 or 12, wherein said fragment may be further linked through its N-terminus to a lauryl-cysteine (LC) residue and/or through its C-terminus to a cysteine (C) residue, or to other residue/s suitable for linking said peptide to adjuvant/s for immunization.
15. A peptide according to claim 14 having the amino acid sequence shown in SEQ ID NO:5 and derivatives thereof, capable of eliciting protective immunity against toxic shock induced by a pyrogenic exotoxin or by a mixture of pyrogenic exotoxins and/or of antagonizing toxin-mediated activation of T cells.
16. A peptide according to claim 14 having the amino acid sequence shown in SEQ ID NO:6 and derivatives thereof capable of eliciting protective immunity against toxic shock induced by a pyrogenic exotoxin or by a mixture of pyrogenic exotoxins and/or of antagonizing toxin-mediated activation of T cells.
17. A peptide according to any one of claims 1 to 16 in the form of a dimer, a multimer or in a constrained conformation.

18. A peptide according to claim 17 having the amino acid sequence shown in SEQ ID NO:7 and derivatives thereof, capable of eliciting protective immunity against toxic shock induced by a pyrogenic exotoxin or by a mixture of pyrogenic exotoxins and/or of antagonizing toxin-mediated activation of T cells.
19. A peptide according to claim 17 having the amino acid sequence shown in SEQ ID NO:8 and derivatives thereof, capable of eliciting protective immunity against toxic shock induced by a pyrogenic exotoxin or by a mixture of pyrogenic exotoxins and/or of antagonizing toxin-mediated activation of T cells.
20. A peptide according to claim 17 which is conformationally constrained by internal bridges, short-range cyclizations, extension or other chemical modification.
21. A peptide according to claim 20 having the amino acid sequence shown in SEQ ID NO:9 and derivatives thereof, capable of eliciting protective immunity against toxic shock induced by a pyrogenic exotoxin or by a mixture of pyrogenic exotoxins and/or of antagonizing toxin-mediated activation of T cells.
22. A peptide according to claim 20 extended at the N-terminus and/or C-terminus thereof with amino acid residue/s identical to those in the corresponding position/s of said pyrogenic exotoxin or with different amino acid residue/s, which may be naturally occurring or synthetic amino acid residue/s.
23. A peptide according to claim 22 having the amino acid sequence shown in SEQ ID NO:10 and derivatives thereof, capable of eliciting protective immunity against toxic shock induced by a pyrogenic exotoxin or by a mixture of pyrogenic exotoxins and/or of antagonizing toxin-mediated activation of T cells.
24. A peptide according to claim 22 comprising the amino acid sequence shown in SEQ ID NO:11 and derivatives thereof, capable of eliciting protective immunity against toxic shock induced by a pyrogenic exotoxin or by a mixture of pyrogenic exotoxins and/or of antagonizing toxin-mediated activation of T cells.

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25. A peptide according to any one of claims 1 to 24 capable of inhibiting expression of pyrogenic toxin-induced mRNA encoded by the IL-2, IFN- $\gamma$  or TNF- $\beta$  genes.
26. A peptide according to any one of claims 1 to 24 capable of eliciting the production of antibodies that block T-cell activation.
27. A pharmaceutical composition for the treatment or prophylaxis of toxin-mediated activation of T cells, comprising as active ingredient a therapeutically effective amount of at least one peptide or derivative thereof according to any one of claims 2 to 25.
28. A vaccine for conferring immunity against toxic shock induced by a pyrogenic exotoxin or by a mixture of pyrogenic exotoxins comprising as active ingredient an immunologically effective amount of at least one peptide or derivative thereof according to any one of claims 1, 3 to 24 and 26.
29. A vaccine according to claim 28 further comprising a suitable immunization adjuvant selected from proteosomes, KLH or alum or combinations thereof.
30. A vaccine according to claim 29 wherein said immunizing adjuvant is a combination of proteosomes and alum or is a combination of KLH and alum.
31. A vaccine according to any one of claims 28 to 30 for enhancing production of antibodies that block T cell activation.
32. A method for treating harmful effects and toxic shock induced by at least one pyrogenic exotoxin comprising administering to a patient in need of such treatment a therapeutically effective amount of a composition according to claim 27.
33. A method according to claim 32 wherein said harmful effect is food poisoning.
34. A method for treating harmful effects and toxic shock induced by at least one pyrogenic exotoxin comprising administering to a patient in need of such treatment a therapeutically effective amount of at least one peptide according to claim 2 to 25.
35. A method according to claim 34 for treating food poisoning induced by a pyrogenic exotoxin.
36. A method for preventing harmful effects and toxic shock induced by a pyrogenic exotoxin or by a mixture of pyrogenic exotoxins comprising

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administering to a patient in need of such treatment a therapeutically effective amount of a composition according to claim 27 or a therapeutically effective amount of at least one peptide according to any of claims 2 to 25.

37. A method for conferring immunity to toxic shock induced by a pyrogenic exotoxin or by a mixture of pyrogenic exotoxins, comprising administering to a patient an effective immunizing amount of a vaccine according to any of claims 28 to 31 or of at least one peptide according to any of claims 1, 3 to 24 and 26.
38. Use of a peptide according to any of claims 1 to 24 and 26 in the preparation of a vaccine according to any one of claims 34 to 38 or of a peptide according to any of claims 2 to 24 in the preparation of a pharmaceutical composition according to claim 27.
39. An antiserum containing antibodies directed against a peptide according to claim 25, which peptide is capable of eliciting the production of said antibodies, preferably in the presence of a suitable immunization adjuvant.
40. An antiserum according to claim 39 wherein said suitable immunizing adjuvant is proteosome, KLH or alum or combinations thereof.
41. An antiserum according to claim 40 wherein said immunizing adjuvant is a combination of proteosomes and alum or is a combination of KLH and alum.
42. An antiserum according to any of claims 39 to 41 which is a domestic animal antiserum.
43. An antiserum according to any of claims 39 to 42 capable of alleviating harmful effects and toxic shock induced by a pyrogenic exotoxin or by a mixture of pyrogenic exotoxins.
44. A method for assessing the efficacy of a vaccine for conferring immunity against one or more pyrogenic toxins comprising determining the ability of serum from an immunized individual to antagonize toxin-mediated activation of T cells.
45. A method according to claim 44 wherein the ability of serum from an immunized individual to antagonize toxin-mediated activation of T cells is determined by measuring the inhibition of expression of pyrogenic toxin-induced mRNA encoded by the IL-2, IFN- $\gamma$  or TNF- $\beta$  genes.

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46. A kit for assessing the efficacy of a vaccine for conferring immunity against one or more pyrogenic toxins comprising determining the ability of serum from an immunized individual to antagonize toxin-mediated activation of T cells by the method of claim 44 or 45.



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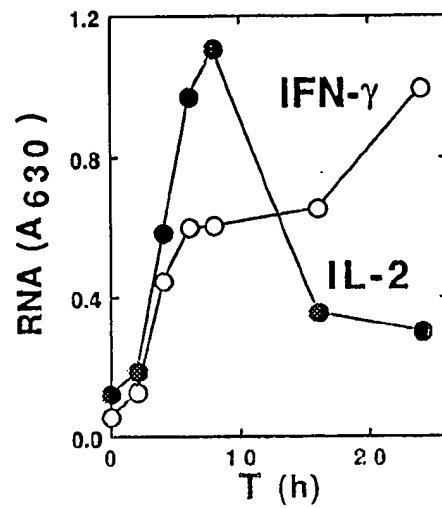


Figure 1A



Figure 1B

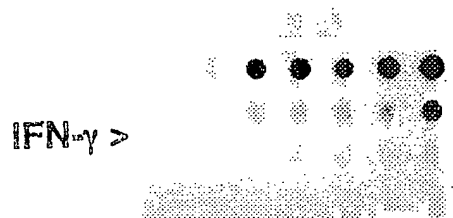


Figure 1C

0 2 4 8 24 h

< IL-2

< IFN-γ

< β-actin

Figure 1D

0 6 12 26 32 48 h

< TNF-β

Figure 1E

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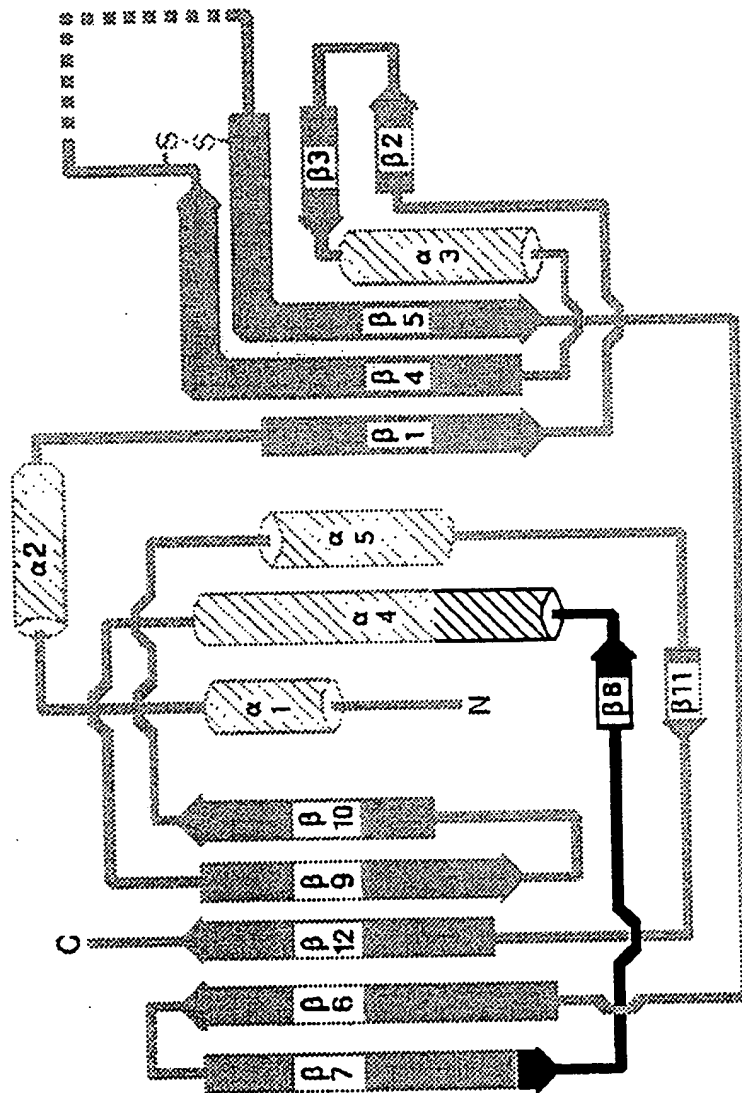


Figure 2

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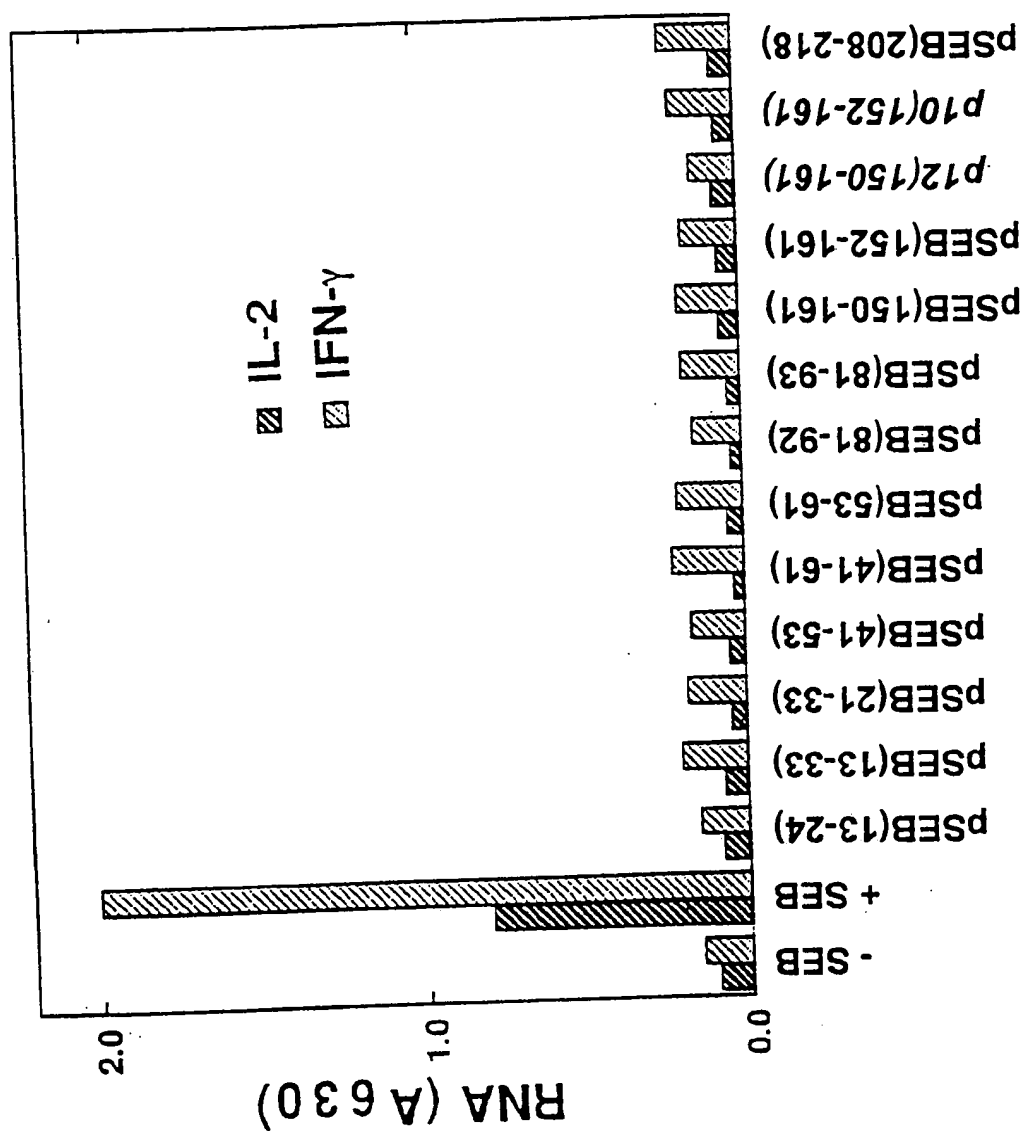


Figure 3

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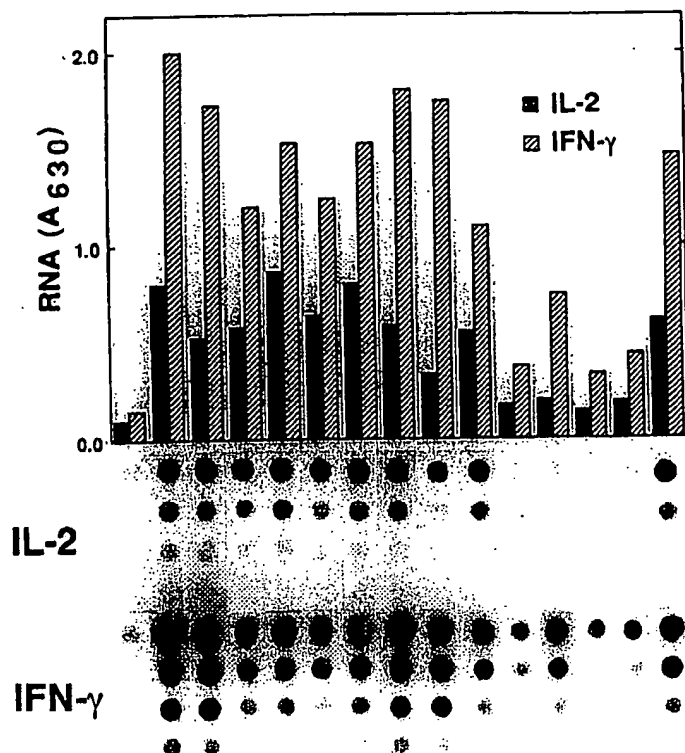


Figure 4A

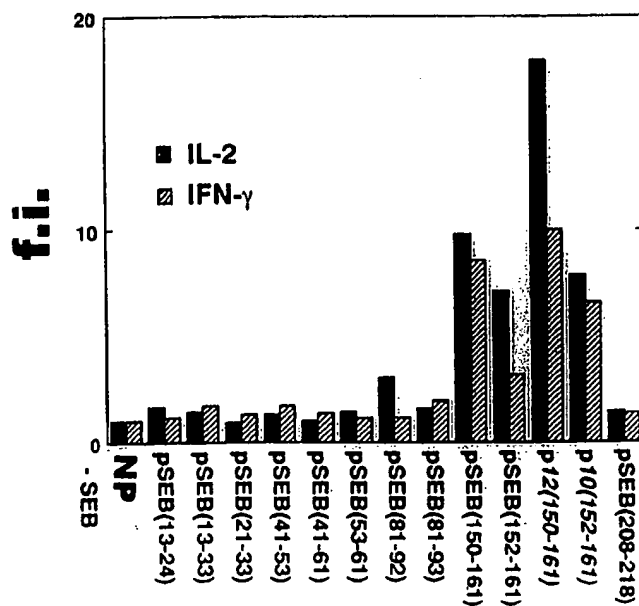
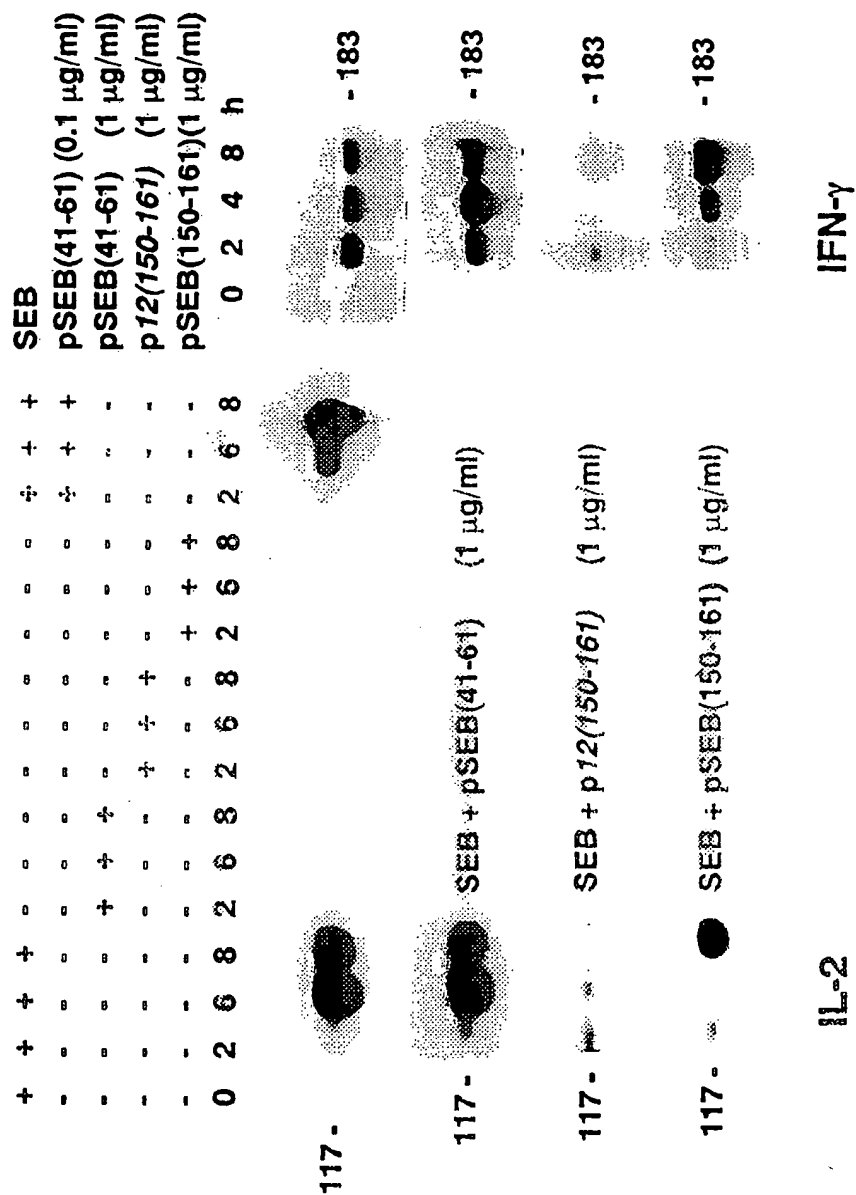


Figure 4B

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## Figures

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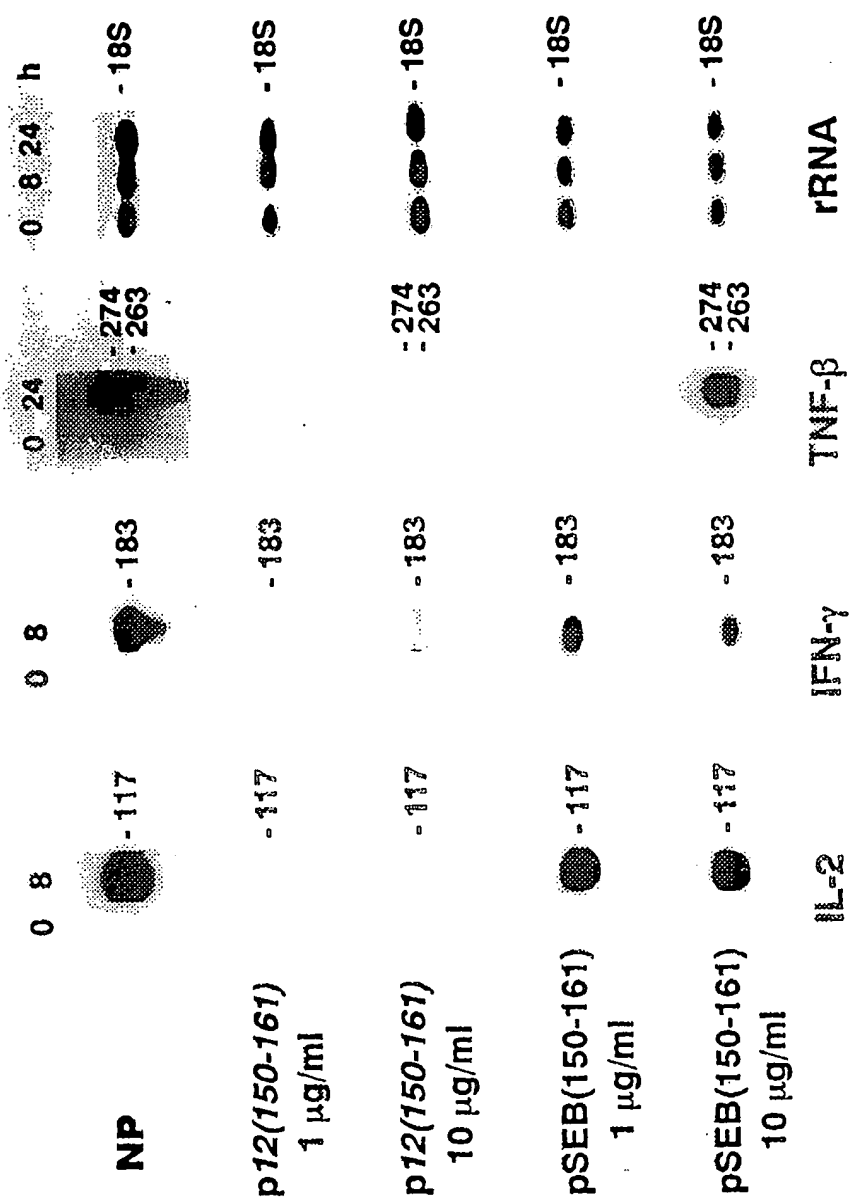


Figure 6

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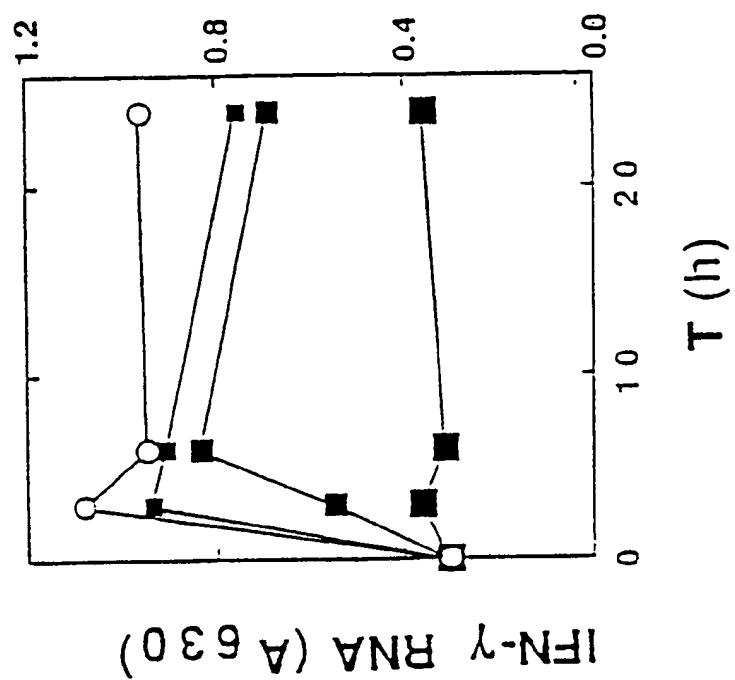


Figure 7B

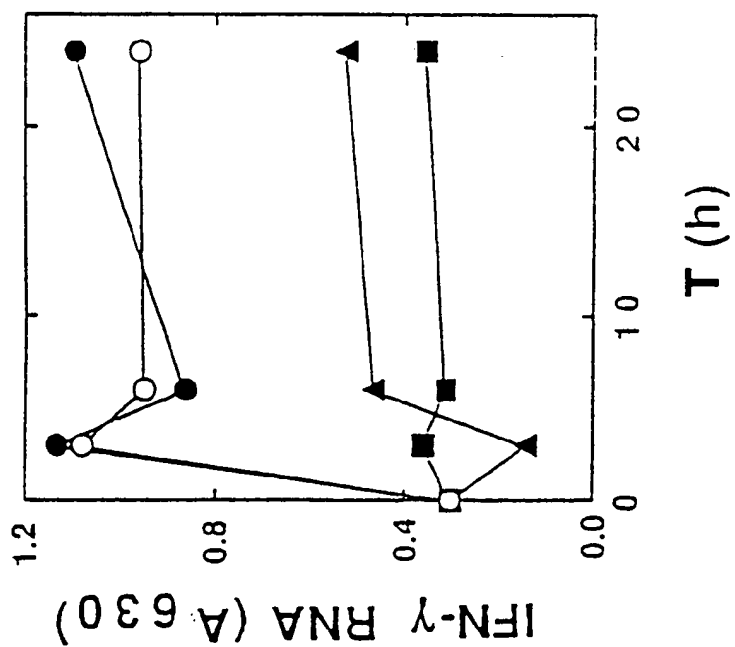


Figure 7A

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Figure 8A

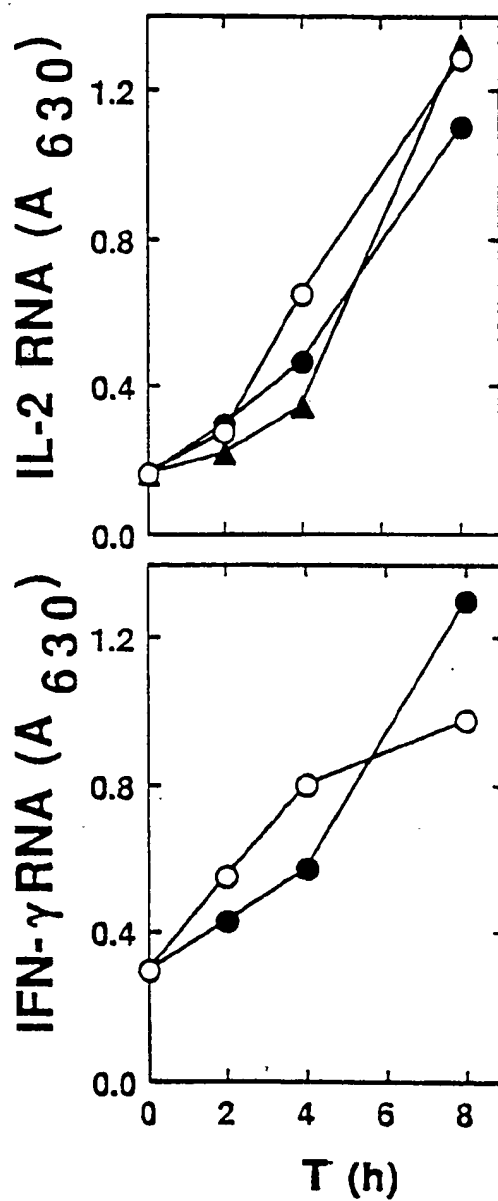


Figure 8B

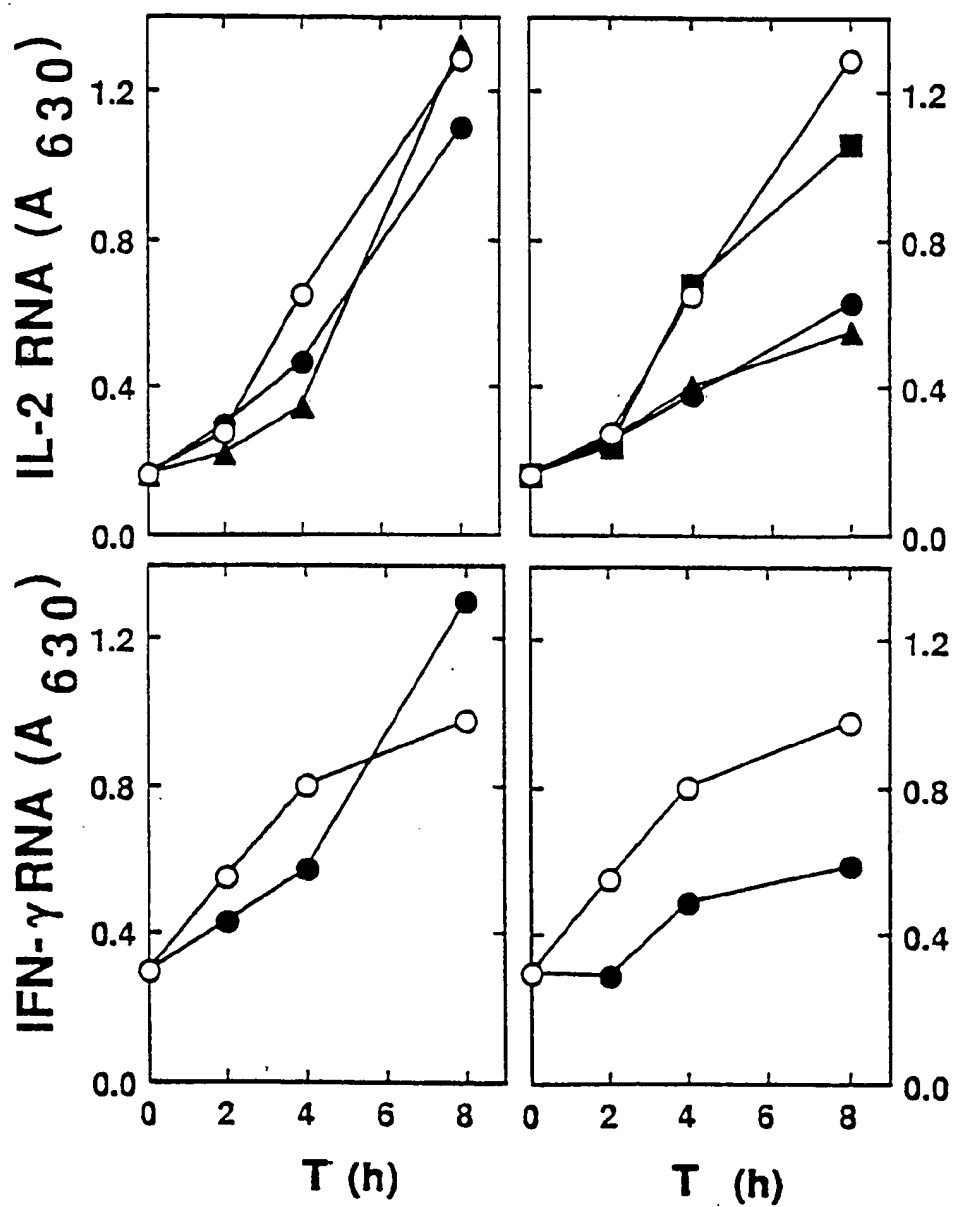


Figure 8C

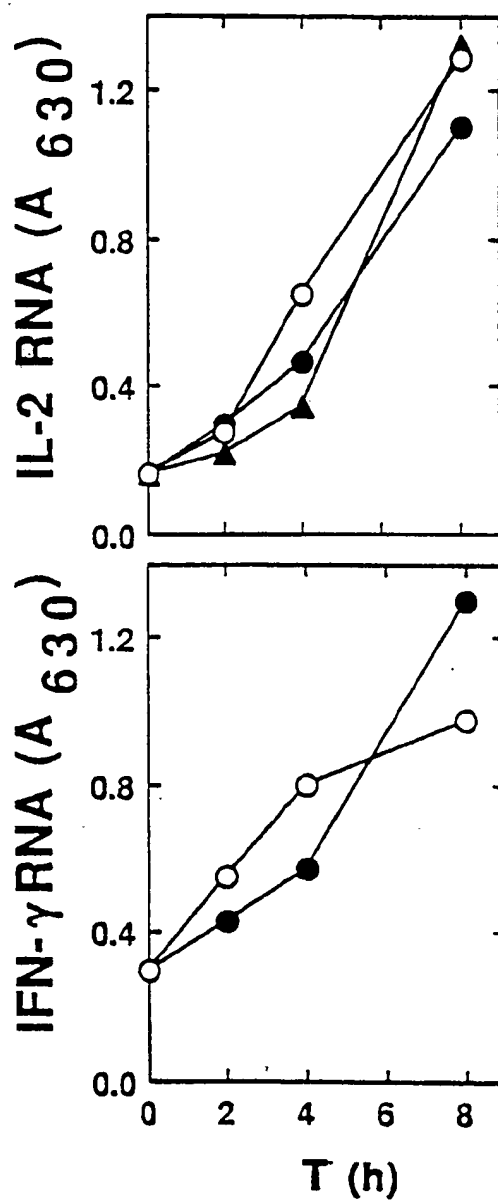
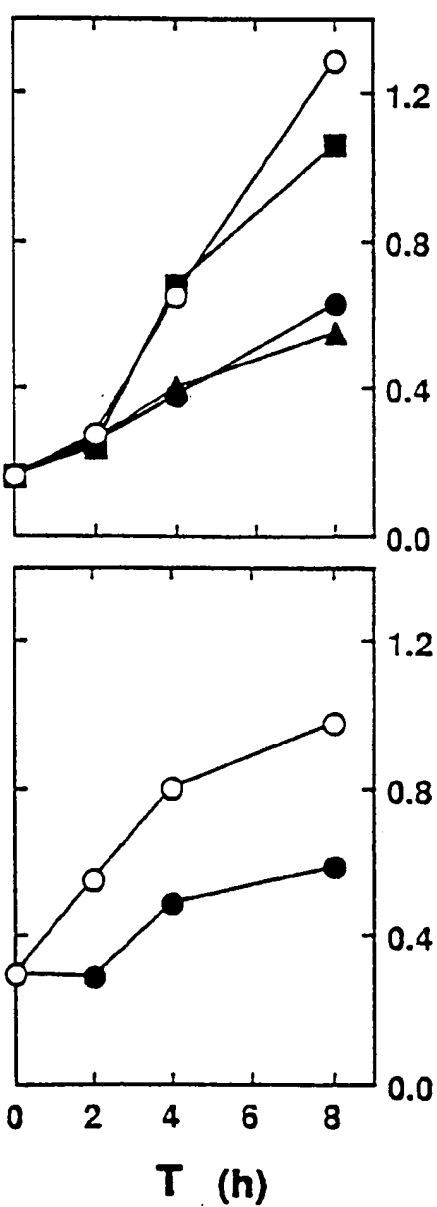


Figure 8D





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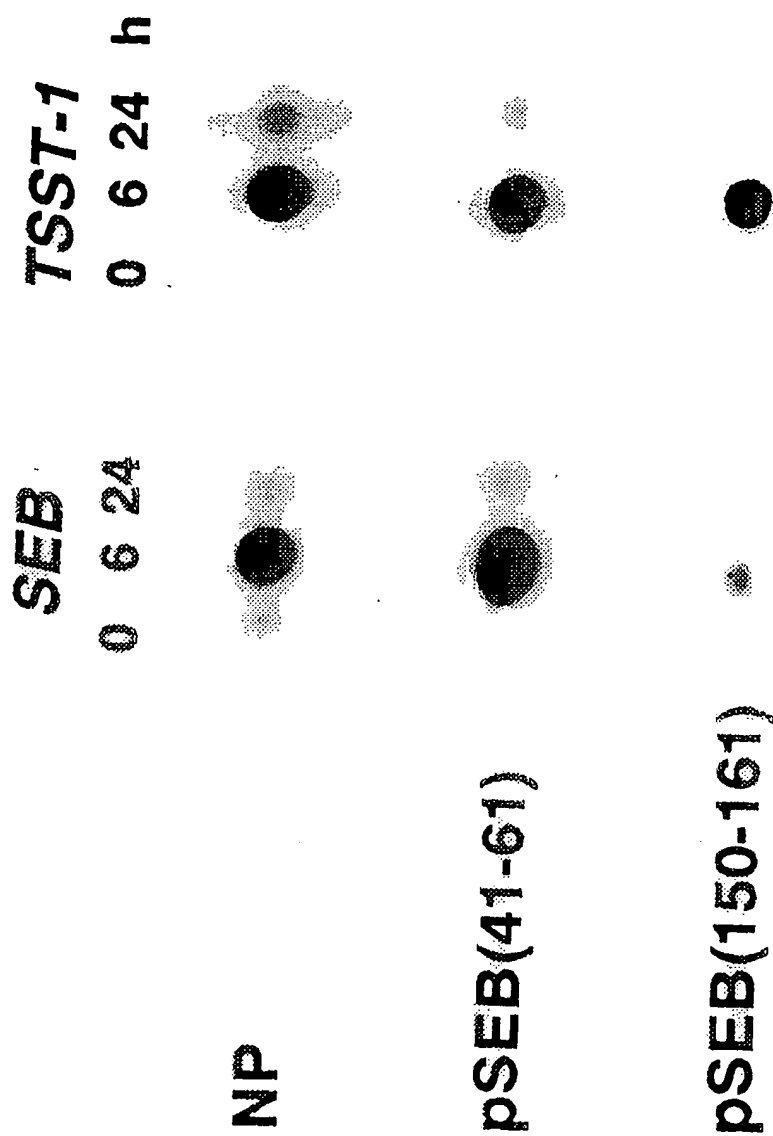


Figure9

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Figure 10A

Figure 10B

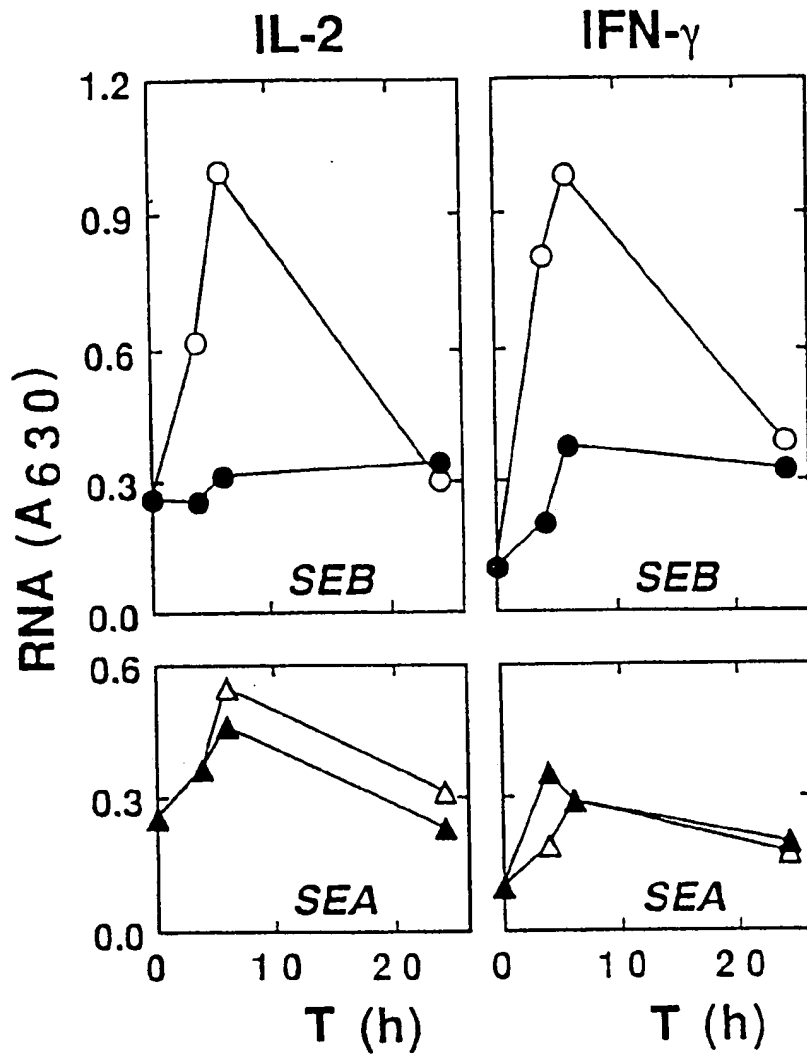


Figure 10C

Figure 10D

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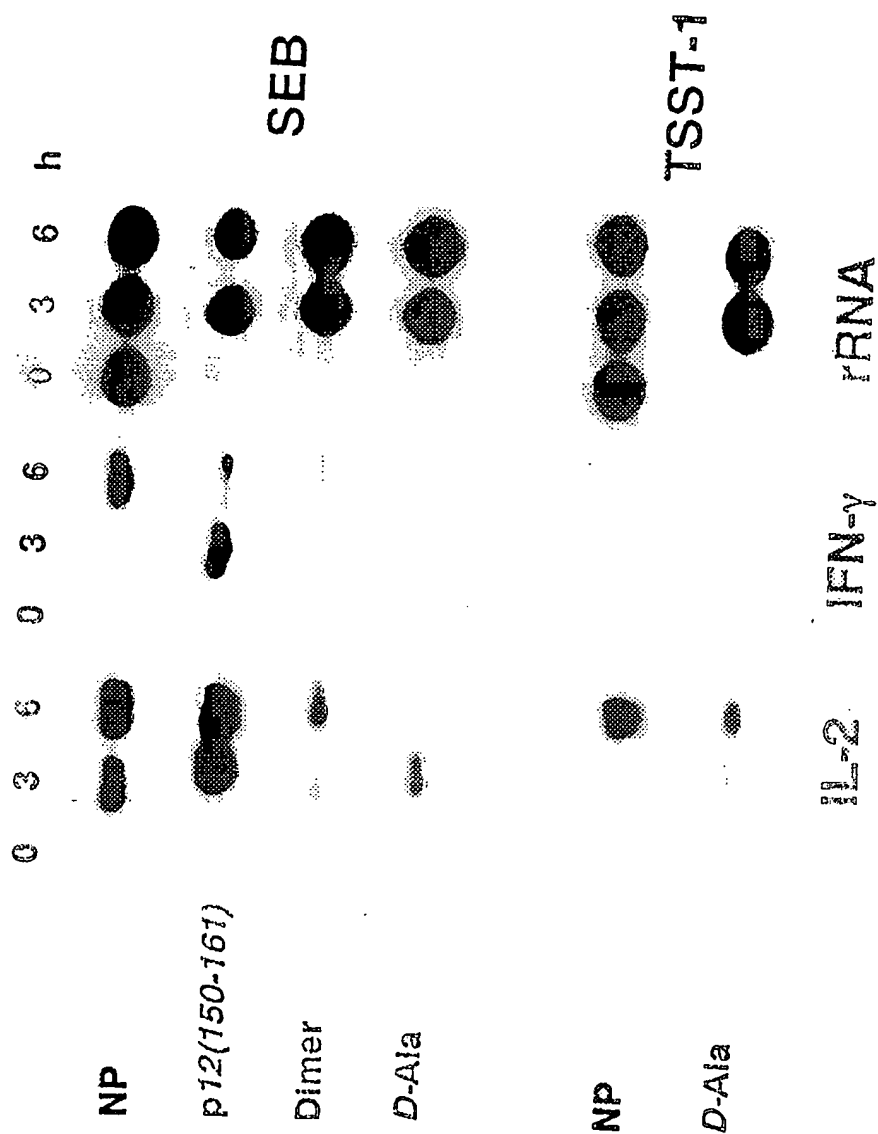


Figure 11

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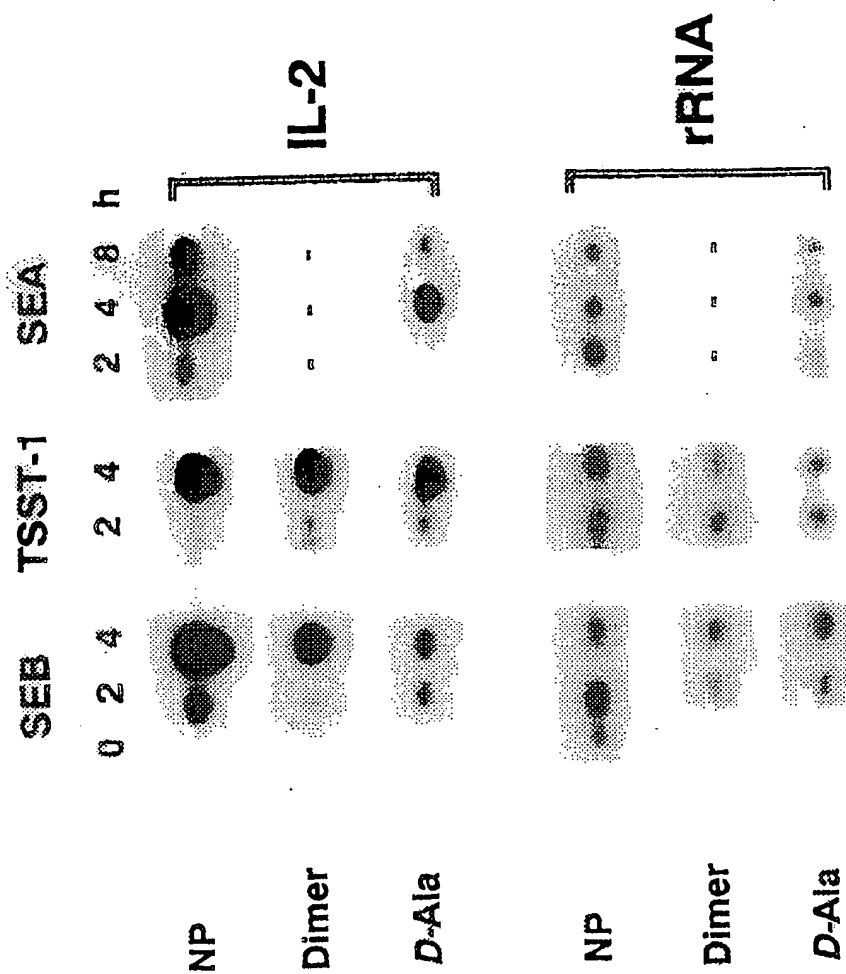


Figure 12

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Figure 14B

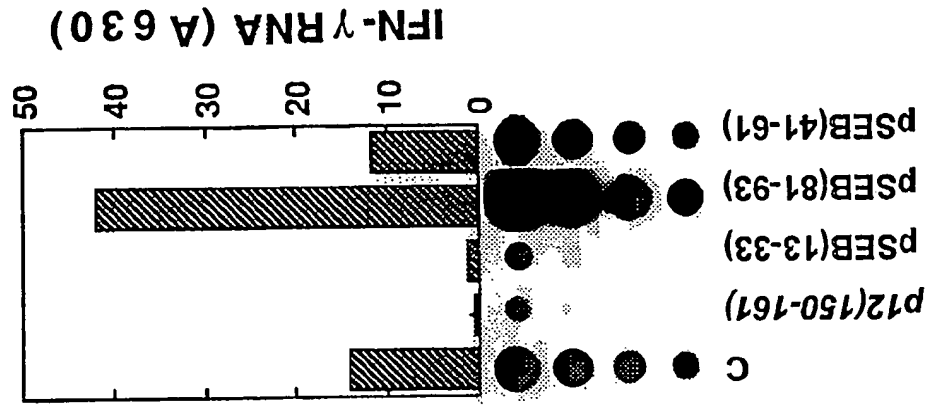


Figure 14A

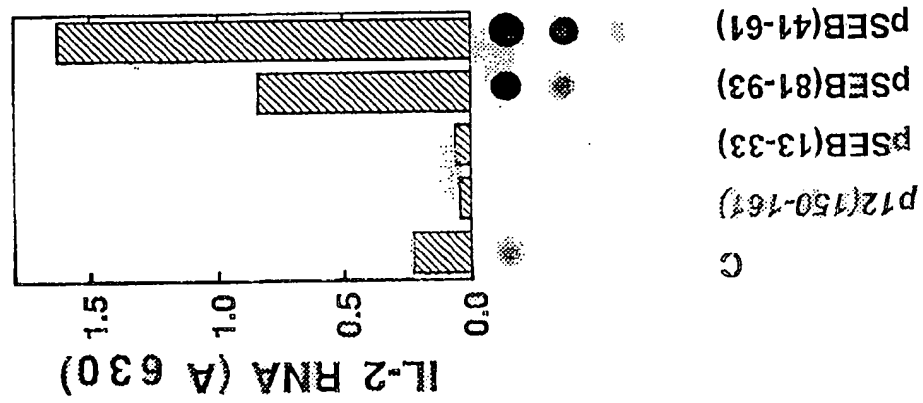


Figure 13B

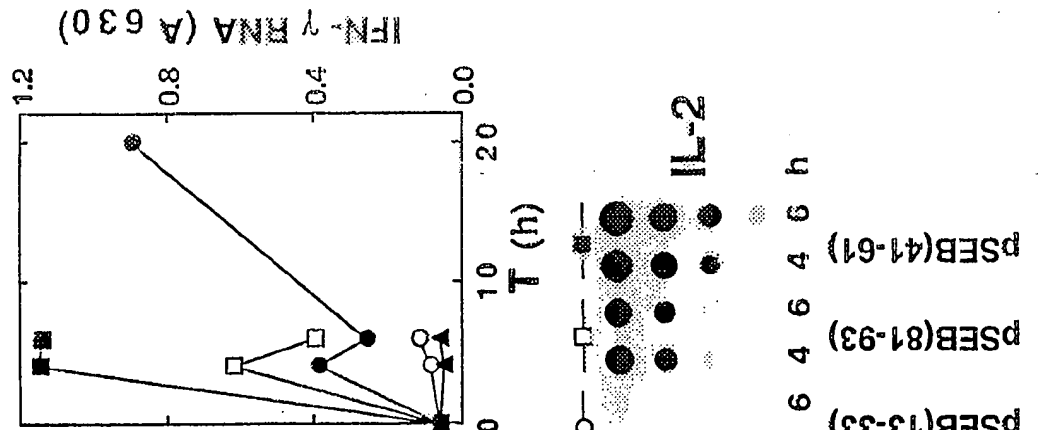
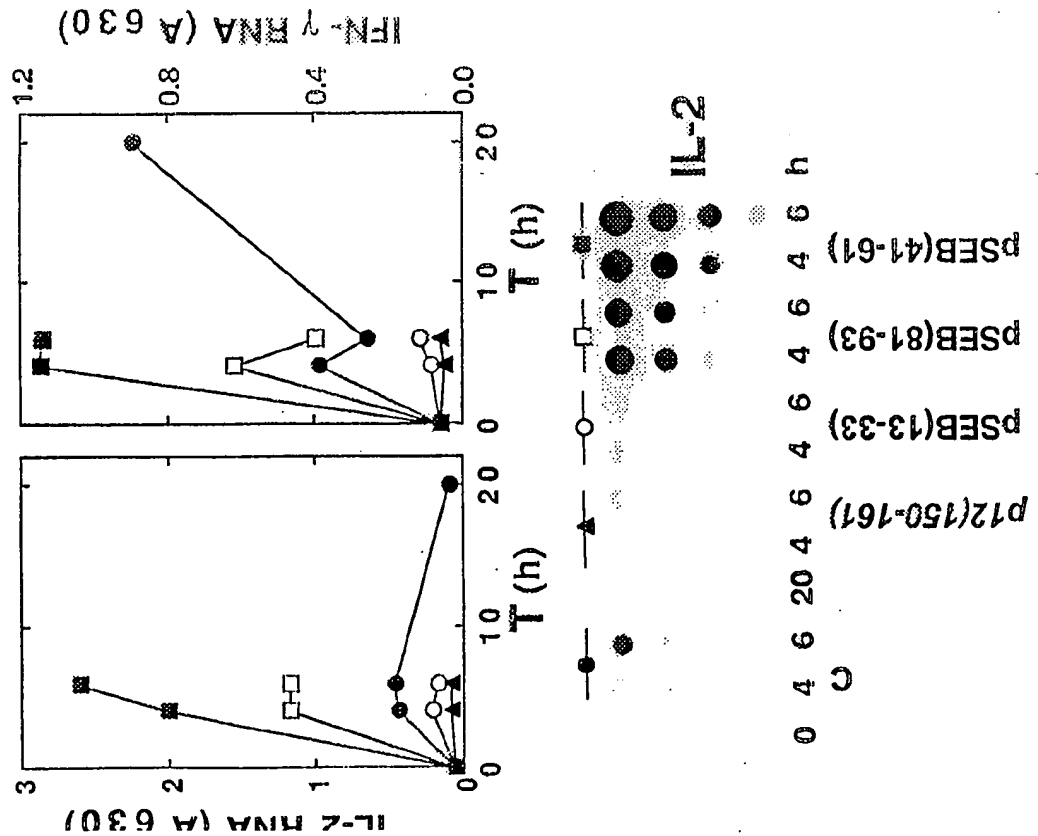


Figure 13A



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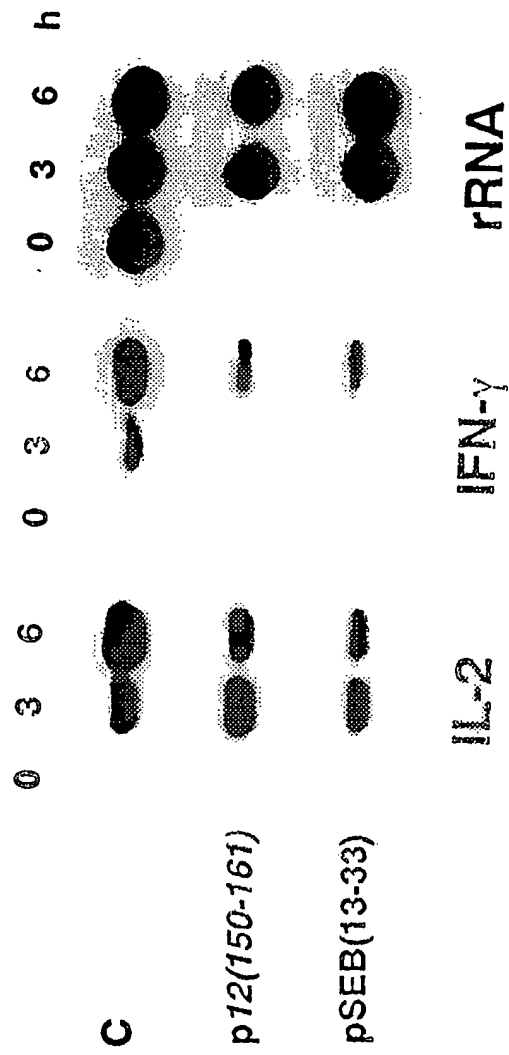


Figure 15

## INTERNATIONAL SEARCH REPORT

Internat Application No

PCT/IL 97/00438

## A. CLASSIFICATION F SUBJECT MATTER

IPC 6 C07K14/31 C07K14/315 A61K38/16 A61K39/085 C07K16/12  
G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M. JETT ET AL: "Identification of Staphylococcal enterotoxin B sequences important for induction of lymphocyte proliferation by using synthetic peptide fragments of the toxin" INFECTION AND IMMUNITY, vol. 62, no. 8, 1994, pages 3408-3415, XP002062764 cited in the application see the whole document see page 3411, last paragraph; figures 2,3; table 1 see page 3409, column 1, paragraph 4 - paragraph 5  --- -/-	1-46



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

Date of the actual completion of the international search

21 April 1998

Date of mailing of the international search report

02. 06. 1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
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Fax: (+31-70) 340-3016

Authorized officer

Cervigni, S

# INTERNATIONAL SEARCH REPORT

Intern 1st Application No

PCT/IL 97/00438

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 40235 A (NEXSTAR PHARMACEUTICALS INC) 19 December 1996 see page 5 see page 17; claim ALL; example 3 ---	1-6,12, 25-46
X	US 5 545 716 A (JOHNSON HOWARD M ET AL) 13 August 1996 *seq ID. 7* see column 1 - column 4 ---	1-6,12, 25-46
X	WO 91 10680 A (TERMAN DAVID S ;STONE JAY L (US)) 25 July 1991 *compare residues 160-170 of SE-A to SE-E* see figure 1 see the whole document ---	1-6,12, 25-46
X	WO 91 12818 A (IMMULOGIC PHARMA CORP) 5 September 1991 see the whole document ---	1-6,12, 25-46
X	LOWELL G ET AL: "MUCOSAL IMMUNOGENICITY AND EFFICACY OF PROTEOSOMES AND PA ADJUVANTS FOR HIV INFLUENZA, SHIGELLA & STAPH. ENTEROTOXIN B (SEB) VACCINES" JOURNAL OF CELLULAR BIOCHEMISTRY, vol. 19A, 5 January 1995, page 259 XP002055657 see abstract ---	28-31
X	PATENT ABSTRACTS OF JAPAN vol. 010, no. 389 (P-531), 26 December 1986 & JP 61 178661 A (TOYOKO CO LTD), 11 August 1986, see abstract -----	28-46



# INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL 97/00438

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 32-37  
because they relate to subject matter not required to be searched by this Authority, namely:  
Although claims 32-37 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

...formation on patent family members

International Application No

PCT/IL 97/00438

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9640235 A	19-12-96	AU 5960096 A	30-12-96
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US 5545716 A	13-08-96	NONE	
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WO 9110680 A	25-07-91	CA 2078003 A	18-07-91
		EP 0511306 A	04-11-92
		US 5728388 A	17-03-98
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WO 9112818 A	05-09-91	AU 7310491 A	18-09-91
		EP 0477307 A	01-04-92
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